SYNTEX, S.A.
KM. 4 CARRETERA CUERNAVACA-CUAUTLA
APARTADO POSTAL 517, CODIGO 62000
CUERNAVACA MOR, MEXICO
TEL. 15-30-00 TELEX 17-3310
DIV. QUIMICA

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CERTIFICATE OF ANALYSIS

MATERIAL ETINIL ESTRADIOL

LOT No. C4-AQ-003 ANALYSIS No. 84 F 1331

SPECIFICATION No. S-75

ANALYSIS	METHODS	RESULTS
APPEARANCE	M-35	White crystalline powder
LOSS ON DRYING	M-19	0.04%
MELTING RANGE	M-7	181.0 - 182.0ºC (As class 1a. USP-XX)
CLARITY OF SOLUTION	M-36	Clear and free of foreign matter
SPECIFIC ROTATION $[\alpha]_D^{25}$	M-8	-28.2º, -28.2º (0.4% in Pyridine)
ULTRAVIOLET $E_{1cm}^{1\%}$ SAMPLE / STANDARD	M-9	69.9, 69.3 at 281 nm 99.2% 70.4, 69.9 at 281 nm
INFRARED	M-11	Consistent with reference spectrum
ASSAY	M-12	99.1%

F-9-001-36

REMARKS:

P.P.

RELEASED BY Dr. Miguel A. Díaz Parra DATE 21-II-85

P. Cabañas

THE EFFECTS OF SEVERAL NATURALLY OCCURRING ESTROGENS ON SAROTHERODON AUREUS (STEINDACHNER) AND THEIR POTENTIAL APPLICATION TO YIELD MONOSEX GENETIC MALES

Paul F. Parks, Dean
Graduate School

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MONOSEX GENETIC MALES

Gary Leo Jensen

Certificate of Approval:

J. R. Snow, Associate
Professor, Fisheries and
Allied Aquacultures

W. S. Shelton, Chairman
Assistant Professor, Fisheries
and Allied Aquacultures

J. A. Plumb, Assistant
Professor, Fisheries and
Allied Aquacultures

E. W. McCoy, Associate Professor
Agricultural Economics and Rural
Sociology

Paul F. Parks, Dean
Graduate School

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THESIS ABSTRACT

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Gary Leo Jensen

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Three naturally occurring estrogens, estriol, estrone and 17B-estradiol were administered perorally to 8-11 mm fry each at concentrations of 30 ug/, 60 ug/ and 120 ug/g diet for both 3 and 5 wk time regimes. Estrogen treatment neither affected survival nor altered growth of experimental fry.

Post-treatment stocking in outdoor concrete tanks revealed no apparent differences in survival between experimental and control groups after 99-148 culture days. Gonadal examination of treated and untreated groups

after harvest revealed no aberrant sex ratios in experimental groups, but there were significant proportions of individuals in all treatment groups with female-like urinogenital papillae. Greater proportions of "atypical" males occurred at higher estrogen dosage levels and in the longest treatment period. No "atypical" males were found in control groups. Sexing of recovered post-treatment mortalities indicated no sexual differential in mortalities. A significant proportion of harvested fish from both experimental and control groups possessed physical anomalies.

Histological examination of gonads revealed no alteration of normal male and female germ cell development, but there were pseudo-oocytes in both treated and untreated groups. No ovotestis condition was observed.

Noneffective alteration of gonads of genetic males by treatments tested, suggested subthreshold steroid concentrations or inadequate treatment duration in relation to the critical period when the gonad completes its sexual determination and differentiation processes.

Early ontological studies revealed that pro-larvae were 5.0 mm after hatching, accepted exogenous feed when 6.5 mm or 3.5 days post-hatching, and absorbed their embryonic yolk sac when 6.5-6.8 mm or 6-7 days-old after hatching. A regression of weight on length and growth curves for laboratory-reared fry are presented.

Economic analysis of estrogen use in potential monosex male production demonstrated its economic feasibility and practicality.

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I. INTRODUCTION

Sarotherodon aureus (Steindachner) belongs to the family Cichlidae and is one of the groups of mouthbrooders which was recently transferred from the genus Tilapia (Trewavas, 1973). Sarotherodon aureus, formerly Tilapia aurea, was confused with and erroneously reported as Tilapia nilotica for several years (Smith-Vaniz, 1968) until Trewavas (1966) distinguished between the two species.

Various species of tilapias are endemic to African waters and have been extensively stocked worldwide in fish ponds where water temperatures are sufficiently warm to permit reproduction and growth. Many have heralded their introduction as a panacea to a cheap source of protein (Atz, 1954; Chimits, 1955; Lin, 1963; Lovshin and Da Silva, 1975). In spite of their precocious and prolific reproduction, other attributes as pond fish are highly desirable. In areas with sustained high temperatures and day length, asynchronous and continuous uncontrolled spawning (Hyder, 1970) produces crops of unmarketable, emaciated and stunted fish which soon negate their good points (Hickling, 1963; Huet, 1970). A high fecundity and the practice of oral incubation assures high recruitment (Lowe, 1955b; McBay, 1961; Dadzie, 1970). For example, Bowman (1974) reported a recruitment of 110, 385 fish/ha in a pond stocked with 2062, 13 g T aurea

after 120 days and Hickling (1963) reported that 14 Tilapia mossambica produced 14,000 offspring in 2.5 mo.

Monoculture of tilapia eventually waned in many areas as their prospering fertility overwhelmed fish farmers' managerial capabilities. Many fish ponds built for tilapia culture were employed for rearing other species or entirely abandoned (Hickling, 1963; Jensen, 1973; Lovshin and Da Silva, 1975).

To combat this undesirable culture situation several stratagems have been evaluated and adopted with varying degrees of effectiveness. These include: culturing fish under crowded conditions or in atypical environments; rearing with a voracious piscivore in polyculture system; utilizing cages as culture enclosures; inducing sterilization; practicing short-term harvests by continuous cropping or stocking yearling fish; and establishing monosex groups for stocking. Some of these techniques are presently experimental while others are being implemented.

Swingle (1957) prescribed crowding as a technique for possible population control due to a "repressive factor" and in (1960) reported no reproduction in T. aurea when stocked in earthen ponds at 40,000 fish/ha. These results could have been due to stocking young of year tilapia which appear to not reproduce until the beginning of their second year at more northern latitudes (Yashouv, 1958).

Culture of T. aurea in high salinity ponds of 36.6 to 44.6 ppt revealed that fish grew at a rate comparable to those cultured in freshwater but did

not reproduce (Chervinski and Yashouv, 1971). This provides promise for coastal zones but has little application to vast inland areas.

The combination culture of a piscivore with tilapia has been effective in controlling tilapia reproduction and resulted in higher A_T values once an efficient carnivore-tilapia ratio is used (Swingle, 1969; Dunseth, 1975). Most areas have endemic piscivores suitable for this approach (De Menezes, 1967; Meschkat, 1967), but a decrease in production from tilapia monoculture is usually experienced (Swingle, 1960; Meschkat, 1967; Semakula and Makoro, 1967). Huet (1970) further stated that the association of tilapias with a predator is a delicate method to apply and results are uncertain due to the varying activity of the predator.

Pagan (1969) reared tilapia in floating cages which was effective in controlling excessive reproduction. Spawning proceeds but fertilization and/or oral incubation is prevented since the eggs pass outside the cage. A complete diet is needed and biological problems inherent when fish are crowded may arise (Pagan, 1970). Cage construction is costly and their longevity is short.

Al-Daham (1970) evaluated the use of irradiation and chemosterilants to sterilize or alter sex ratios in T. aurea. Cobalt-60 gamma rays, decreased the gonadosomatic index in both sexes when compared to controls but produced no deviation from normal sex ratio. Both chemosterilants (metepa and tretamine) examined, inhibited brood production from 2 to 3 mo and produced severe testicular atrophy but slight effects on ovaries. In a similar

approach, Hyder (1972) employed the use of methallibure to inhibit complete spermatogenesis, vitellogenesis and gonadal steroidogenesis in Tilapia nigra. He reported prevention of transformation of spermatogonia into spermatocytes and sperm release but spermatocytes to spermatozoa development continued while inhibition of vitellogenesis and resorption of yolk-containing ova occurred in females. Similar results with methallibure were obtained by Dadzie (1972) in T. aurea and T. mossambica. Growth rate was not affected by treatment and oral administration resulted in more rapid effect than the drug addition to water. This synthetic drug inhibits pituitary-gonadotropic function, thus achieving a form of physiological hypophysectomy (Hoar et al., 1967). These sterilant methods are still experimental and their application to practiced fish culture questionable.

While subsistence or rural fish farmers probably evolve their own simplistic tilapia culture methods (Chimits, 1955, 1957; Mortimer, 1967), irrespective of population control, commercial food fish producers with intentions of growing tilapia, need an economically practical solution. The production or stocking of monosex seedlings presently affords the most promising approach in curtailing tilapia reproduction and producing high yields (Hickling, 1960, 1971; Meschkat, 1967; Bardach et al., 1972; Bard et al., 1974; Lovshin and Da Silva, 1975).

Superior growth of male tilapias enhances the practice of all-male stocking (Kirk, 1972). At equivalent stocking rates and pond conditions with males and females cultured in separate ponds, faster male growth was

reported by van Someran and Whitehead (1960) in T. nigra, Yashouv (1958) in T. nilotica and Guerrero and Guerrero (1975) in T. mossambica. Pruginin and Shell (1962) revealed only male T. aurea reached desired harvest size during one rearing season and Dunseth (1975) noted T. aurea males attained twice the average daily weight gain of females when stocked in communal ponds. T. nilotica males were reported by Bard (1973) to surpass weight gain of females by 50-60 g after 113 growth days. Avault and Shell (1968) reported male hybrid tilapia grew 2.3 times faster than female hybrids when stocked in 0.002 ha concrete tanks. T. mossambica male growth excellence was also demonstrated when separated sexes were reared in cages (Inland Fisheries Project, Philippines, 1975).

Monosex tilapia populations can be obtained by means of manual or mechanical sexing, interspecific tilapia crosses, some of which produce monosex progenies, and functional or phenotypic sex reversal by administration of heterosexual steroidal hormones.

Tilapia fingerlings of 7.0 cm can be sexed externally by examination of the genital papilla (Yashouv and Hefetz, 1959). Sexual differences in genitalia are discussed by Yashouv and Hefetz (1959) and illustrated by Lowe (1955a) and Maar et al. (1966). Pruginin and Shell (1962) stated that hand-sexing T. aurea yields an average of 500 to 800 sexed fish per man-day and only about one half of these are males. Mires (1969) thought that T. aurea should be manually sexed only after reaching 80 g, or approximately 16-17 cm. Hickling (1963) emphasized the disadvantages in hand-sexing as

tedious, time consuming, unreliable and wastefullness of females. Chervinski (1965) suggested separating sexes in T. aurea by sexual differences in the shape of the dorsal and anal fins common to fish larger than 60 g (14 to 15 cm). Huet (1970) stated mistakes in hand-sexing are inevitable and this method can only be used by experienced fish farmers, aided by very conscientious personnel.

Pruginin and Shell (1962) devised a slatted grader to mechanically separate sexes in T. aurea of 13 g by sexual differences in body thickness. In some cases, they were able to separate with reliability 95% of males in tested groups. Fish sexed must be of approximately the same age and the sample of fish measured must represent the population to be sexed. Bard et al. (1974) also mentioned employment of mechanical graders to separate sexes of T. nilotica when they reach 100 g.

Since Hickling (1960) first reported his discovery of all-male offsprings produced from an interspecific tilapia cross, investigators have demonstrated similar findings in five different tilapia interspecific matings (Pruginin et al., 1975). Six crosses have produced 100% male hybrids, but three occasionally produce a small proportion of females (Pruginin et al., 1975). These same authors stated that in Israel, commercial production of monosex hybrid fry has limited use because of the difficulty in maintaining pure parental stocks that consistently produce 100% male offsprings. In addition, F_1 hybrids are fertile and taxonomy and identification of tilapia species are confusing, thus duplication of a desirable hybrid may prove

difficult. Hickling (1968) discussed further difficulties in fish hybridization work. Lovshin et al. (1974) successfully propagated all-male hybrids for several years by crossing male Tilapia hornorum with female T. nilotica. Success can be attributed to obvious visual differences between species and to a conscientious, technical breeding program.

Yamamoto's pioneering work in functional sex reversal, reviewed by Yamamoto (1969), demonstrated that complete and functional sex reversal could be induced in both genetic sex directions in teleosts by oral administration of sex hormones. He further showed that when the homogametic sex fish was sex reversed and crossed with normal fish of similar sex genotype, monosex offspring were produced. Clemens and Inslee (1966) produced all-female progeny in T. mossambica by mating functionally sex reversed females, treated with an androgenic sex hormone, with untreated females, providing evidence for female homogamety. Recently, Guerrero (1975) by treating T. aurea fry with several androgens, achieved 100% sex reversal in genotypic females and in subsequent matings of treated and untreated fish established female heterogamety and suggesting male homogamety.

Guerrero (1975) stated that sex reversal appeared to be a practical method for producing monosex male tilapia by treatment with androgens. Furthermore, it was seemingly inexpensive, thus reinforcing its economic feasibility (Guerrero, 1974). Employing androgens with T. aurea,

necessitates continuous fry collection and treatment with consistent and sufficient production of 8.0 to 11.0 mm fry which is a limiting factor for all-male production. Growth and yields of treated groups composed of approximately 50% female genotypes would likely be inferior to those of all-genotypic male groups if male growth superiority has a genetic basis as suggested by Fryer and Iles (1972). Guerrero (1975) also discussed the questions concerning the human consumption of fish treated with sex hormones.

Recently, Nakamura and Takahashi (1973) induced complete feminization of the gonads in genetic males of T. mossambica using a synthetic estrogen.

To further assist tilapia culturists by employing recent results obtained with sex hormones, the use of estrogens on homogametic S. aureus males was proposed.

This study was conducted to evaluate the effectiveness of three naturally occurring estrogenic hormones (estriol, estrone and 17B-estradiol) as potential agents to induce complete and functional sex reversal in male genotypic fry of S. aureus. In the event of success, these sex-reversed males would, in future studies, be mated with normal males in the hope of producing all-genotypic male offsprings.

II. REVIEW OF LITERATURE

Status of estrogen research in teleosts

Several investigators have evaluated the use of estrogens or materials with estrogenic potency concerning their effectiveness in altering primary and secondary piscine sexual characters. Unfortunately, results are sometimes paradoxical and difficult to interpret (Reinboth, 1970). The effects of estrogens on fishes have been investigated far less than the reported research for androgens and with more discordant results (Dodd, 1960). In addition, the effects in fishes are more difficult to interpret than those obtained in amphibia and birds (Ashby, 1957). Pickford and Atz (1957) expressed that results of administering heterologous sex steroids to embryonic fishes are complicated and not decisive, making a brief but balanced summary difficult and dependent on the analysis of scores of papers.

Attention has been focused primarily on aquarium fishes in the families Cyprinodontidae and Poeciliidae due to their marked sexual dimorphism, ease of breeding and handling in laboratories, and presence of sex-linked gene markers (Yamamoto, 1953; Bacci, 1965). Recently, research has been directed toward several species in the family Cichlidae (Hackmann and Reinboth, 1974), some of which have commercial importance as food fishes,

and respond positively to estrogen treatment (Eckstein and Spira, 1965; Nakamura and Takahashi, 1973).

Discovery and nature of estrogens

Allen and Doisy (1923, cited in Fieser and Fieser, 1959), first demonstrated the existence and activity of estrogenic hormones in ovariectomized rats. The following estrogens were isolated and identified in the years indicated; estrone (1929), estriol (1930) and estradiol (1935) (Fieser and Fieser, 1959). Various hormones differ structurally by their attached side chains. Their biosynthetic steps are catalyzed by enzymes via cholestrol to pregnenolone followed by biotransformation of pregnenolone into the various estrogens (Baird, 1972). Testosterone of androgenic potency is converted to estrone or 17B-estradiol in female mammals (Dorfman and Ungar, 1965) with the mammalian ovary producing mainly estradiol of which estrone and estriol are estrogenetically active metabolites (Fieser and Fieser, 1959). Eik-nes (1969) diagrammatically presented the pathways and products involved in biotransformation of pregnenolone in vertebrate gonads while Hoar (1962, 1963) demonstrated the effects of the gonadal hormones in teleostean reproduction.

Occurrence and significance of estrogens in teleosts

Van Tienhoven (1968) listed the various estrogens extracted from the ovary or blood in several fish species, while Katz et al. (1971) stated most workers report presence of estriol, estrone, and 17B-estradiol among the

various steroids extracted from teleostean ovaries. But they reported only estrone and 17B-estradiol from the ovaries of T. aurea, which exemplifies the variability in types and proportions of estrogens between different species (Hoar, 1965).

Disagreement exists in the locus of hormone production in the fish ovary, but their elaboration probably occurs in the corpora lutea and/or granulosa cells (Hoar, 1965). The existence of endocrine tissues in mature gonads and their influence on sex characters is well documented (Hoar, 1965), but it is unknown which hormones, if any, are produced in undifferentiated gonads of teleosts (Reinboth, 1972a).

Yamamoto (1953, 1958) used his success in sex reversal of Oryzias latipes (medaka) with steroid sex hormones to support his opinion th hormones or hormone-like substances are produced by embryonic go s and act as sex inductors. He further stated (1962) that these action-substances may act either (a) directly upon the indifferent germ cells or (b) through interaction with diffusively distributed androgenic and gynogenic somatic tissues, if such be present in medaka. Onitaka (1972) also concluded from experiments with medaka, that sex hormones were natural sex inducers; androgens as andro-inducers and estrogens as gyno-inducers, similar to the non-steroid inductor theory proposed by Witschi (1957) for higher vertebrates. The hypotheses on steroids as sexagens received early support from the bovine freemartin phenomenon discussed by Lillie (1916, 1917). Satoh and Egami (1972) stated that no sexual

differences in somatic elements are seen at hatching in medaka, yet morphological sex differentiation of germ cells occurs at this time. Their results suggested involvement of an intracellular mechanism in sex differentiation. Dodd (1969) further stressed the opinion that genetically mediated inductor substances are thought to be secreted by certain cells of the gonadal stroma that favor development of spermatogonia in the embryonic piscine gonad. Recently, Satoh (1[illegible]), using electronmicrosopy, reported that sex differentiation of germ an[illegible]atic cells in gonads of medaka precedes the differentiation of stero[illegible]-secreting endocrine cells; thus somewhat supporting the intracellular supposition and negating the sex hormone inductor theory. However, when injecting estrogens into fertilized eggs of medaka, results indicated their selective incorporation into the differentiating gonads and their action as sex inducers (Hishida, 1964). Witschi and Dale (1962) stated that the discovery that sex steroids are produced by larval frogs at or immediately after sexual differentiation of gonads seems to encourage the notion that sex hormones might actually be natural sex inductors.

Larval gonadal development and sex reversal

Unlike most vertebrates whose gonads arise in the dorsolateral lining of the peritoneal cavity and have a double origin developing from cortex or medulla to form the ovary and testes, respectively, cyclostomes and teleosts develop their entire gonad directly in peritoneal epithelium which corresponds to only the cortex of other vertebrates (D'Ancona, 1945; Hoar,

1969). Fertilization determines the genotypic sex by the pairing of sex chromosomes and autosomes with their male and female sex-determining factors, chromosomal or genic (Harrington, 1974) but the phenotypic gonadal sex is not necessarily permanently decided at this time (Yamamoto, 1969). Permanency in genetic constitution and plasticity in phenotypic sex are demonstrable in matings of sex-reversed fish with normal fish of known genotypes and evaluation of the resultant offsprings (Yamamoto 1955, 1961; Yamamoto and Kajishma, 1968; Clemens and Inslee, 1968; Guerrero, 1975).

The fact that the larval piscine gonad undergoes an indifferent or undifferentiated stage in development, possesses bipotential or ambivalent capability, and is labile (D'Ancona, 1945; Lowe and Larkin, 1975), help explain the success of functional sex reversal. Yamamoto (1953) described the indifferent period in O. latipes as occurring prior to sex differentiation as did Wolf (1931) in Xiphophorus maculatus, Johnston (1951) in Micropterus salmoides, Ashby (1957) in Salmo trutta and Eckstein and Spira (1965) in T. aurea. It is known that much, or even all, of the gonadal development in teleosteans occurs after hatching (Eckstein and Spira, 1965). The indifferent gonad, irrespective of the sex genotype is ambivalent as regards its future mode of differentiation, viz., has potentiality in differentiating into either a functional ovary or testes in birds (Willier, 1939), amphibia (Chang and Witschi, 1955; Mikamo and Witschi, 1963), mammals (Mittwoch, 1969) and fishes (van Tienhoven, 1968).

Modes of estrogen administration and their significance

Administration of naturally occurring and synthetic estrogens has been varied, producing diverse effects. Methods of estrogen treatment have involved; mixing with diets or per os (Berkowitz, 1941; Yamamoto, 1953, 1968; Sanico, 1975), injecting intraperitoneally (Baldwin and Li, 1945; Egami, 1954; Vallowe, 1957), rearing fish in estrogen-water solution (Cohen, 1946; Ashby, 1957; Reinboth, 1972b) and implanting subcutaneously (Egami, 1955a, b). Hishida (1964) even injected estrogens into fertilized fish eggs. Reinboth (1969) expressed that the paradoxical effects observed in the use of sex hormones in cases where an atypical action has been reported, might depend on hormone concentration, but the method of steroid administration may also affect results.

The manner of administration appears to influence the potency of different estrogens. Fieser and Fieser (1959) reported estradiol administered subcutaneously to rats was 8 times as active as estriol, but when administered orally, estriol was 5 times more active. An explanation was possibly presented by Heller (1940) who stated that orally administered estriol is more potent than estrone and estradiol because it is only mildly affected by the liver, whereas the latter are greatly destructed or inactivated. Hishida (1964) with the medaka, calculated that the estrone effect on sex differentiation had 10 fold potency when given parenterally vs. orally.

Whether an estrogen is synthetic or naturally occurring influences estrogenic potency which also varies depending on the mode of

administration. White et al. (1973) reported that naturally occurring estrogens are most active when administered parenterally, while the most potent orally active estrogen is the synthetic compound, ethinylestradiol, which has approximately 10 times the activity of estrone when given per os. Diethylstilbestrol (DES), another synthetic product, has about 3-5 times the estrogenic potency of estrone when administered orally (White et al., 1973). Fieser and Fieser (1959) stated that ethinylestradiol has an oral activity potency of 12, 13 and 2.5 times that for estradiol, estrone and estriol, respectively, but was equal to estradiol when administered subcutaneously. In sex reversal potencies of genetic male medakas, estradiol and DES had potencies 3.5 and 2.7 times as high as that for estrone, respectively (Yamamoto, 1963), while estriol was 0.045 and 0.15 as potent as estradiol and estrone, respectively (Yamamoto, 1965), when all were administered perorally. It appears that the higher oral activity of the synthetic compounds is due to their superior stability in the gastrointestinal tract and the liver (White et al., 1973).

Contributing factors for success in estrogen treatment

To successfuly obtain sex reversal, Clemens and Inslee (1968) stressed the need of careful feeding and clean conditions in treatment containers to assure consistent administration of hormones per os. Yamamoto (1969) stated that two conditions should be fulfilled for achieving sex reversal in differentiated gonochorists: (1) the heterologous sex hormone should be given starting with the stage of

indifferent gonad and continued throughout the stage of sex differentiation and (2) adequate dosage levels of hormones should be used. The direction of sex reversal may be determined by the kind of hormone acting, the composition of gonad rudiment and the time of action of sex hormones (Willier, 1939).

Whether steroids used in various experiments are identical with ones that occur naturally in fishes, or how similar they may be, has critical bearing on the significance of results obtained (Atz, 1964). Estrogens are predominantly feminizing in action in fishes, but it has been shown that closely related estrogens may have a completely different effect from each other and from that which they show in mammals (Dodd, 1960). Aronson and Holz-Tucker (1947) and Levy and Aronson (1955) hypothesized that hormones secreted by ovaries in Tilapia macrocephala are dissimilar to mammalian estrogens and not equivalent to the commonly available synthetic estrogens. Yamamoto (1969) simply expressed that it is not known whether all the sex hormones of adult fishes are identical to those of mammals.

Use of estrogens in non-cichlid fishes

In pioneering studies, Berkowitz (1937, 1938) demonstrated that male secondary traits and spermatogenesis were suppressed when Poecilia reticulatus (guppy) was fed tablets containing estriol and estrone from birth to 1 - 5 mo. Reappearance of normal secondary sex characters occurred after suspension of treatment and transfer to freshwater.

Berkowitz hypothesized effects were due to either the direct effect of estrogens on male gonad or an inhibition of hypophyseal activity. In subsequent work (1941), he increased his dosage levels and obtained ovotestes, and with longer treatment duration, a more complete (90%) and consistent sex reversal. He further injected alpha-estradiol benzoate, estradiol-17 propionate and stilbestrol (DES) into sexually immature guppies and reported less complete and frequent gonadal modification. Egami and Ishii (1962) established a hypophyseal-gonad relationship and estrogen interference with the secretion of gonadotropins from the adenohypophysis. Miyamori (1964) obtained complete feminization in the guppy with treatment of ethinylestradiol at 125 ug/g diet.

Induction of ovotestes in adult male Xiphophorus helleri (swordtail) by injecting alpha-estradiol benzoate retroperitoneally was disclosed by Baldwin and Li (1945) with two injections per week for 24 wk. Vallowe (1957) similarly obtained ovotestes in the swordtail through weekly injections of estradiol benzoate into 49 to 55 day-old specimens for 9 wk. Essenberg (1926) claimed that the sex in the swordtail is determined and controlled by sex hormones derived from the gonads and that any agent or condition which tends to decrease the capacity for hormone secretion becomes an immediate factor in sex reversal.

Cohen (1946) maintained 2 week-old Xiphophorus maculatus (platyfish) in water containing alpha-estradiol benzoate for 8 to 20 wk and

noted suppression of spermatogenesis beyond spermatocyte stage and induction of ovotestes in some cases. Testes in 20 wk treatment were similar to those in controls and feminized males mimicked female courtship behavior when introduced to untreated males. Later, Tavolga (1949) reported treatment with estradiol benzoate was inhibitory on testis and suppressed gonopodium development while alphaestradiol produced no gonadal changes in males platyfish less than 18 mm, but stimulated testes in males greater than 19 mm that also possessed well-formed gonopodia. Hybrid Xiphophorus injected with estradiol had destruction of spermatophores, a temporary suppression of spermatogenesis, but no modification in secondary sex features (Taylor, 1948).

Ashby (1957) reported a strong inhibition in the development of germinal tissue and lack of evidence in sex reversal when S. trutta alevins were reared in tanks containing estradiol renewed daily. Conversely, Padoa (1937, 1939, cited by Ashby, 1957) obtained induction of sex reversal in a related species, Salmo irideus, treated with estrone dissolved in culture medium.

Impetus to the potential importance of heterotypic sex hormone treatment to teleosts was realized when it was convincingly demonstrated that estrogens (estrone and DES) administered perorally during the larval life of O. latipes (medaka) produced complete, functional reversal of sex differentiation in genetic males (Yamamoto 1953, 1957, 1959 a,b,

1962). Yamamoto worked with a medaka variety that possessed a sex-linked gene for body color (Aida, 1921), thus allowing easy detection of genotypic females (white) and males (orange) without reliance on secondary sexual traits. He experienced similar sex inversion success with 17B-estradiol (Yamamoto, 1963) and estriol (Yamamoto, 1965). A linear relationship between estrogen dosage levels and resultant percentages of sex reversal was shown (Yamamoto, 1959a), including the occurrence of intersexes at subthreshold levels (Yamamoto, 1957).

Yamamoto (1963) further revealed that mating estrone-induced females with normal males yielded unusual (YY) males which when crossed with normal (XX) females sired all-male progeny with normal male (XY) genotype. By treating two consecutive generations with estrone, Yamamoto (1967) produced (YY) females. Equally important, Yamamoto (1968) stressed the permanency of sex reversal alteration in medaka where fertility for the full life span is retained.

Egami (1954) also working with the medaka, demonstrated that oviposition in females was inhibited when estrone benzoate was either injected or implanted subcutaneously. Egami (1955 a,b) further tested estrone benzoate by injection, subcutaneous implantation and oral administration in adult males and obtained no complete sex reversal, but formation of ovotestes. Results indicated that certain doses of estrogen interferred readily with spermatogenetic acitivity but later activity is recovered, that fish with recovering testes were prone to

ovotestes formation and that ovotestes produced by different modes of administration were the same in structure.

Hishida (1964) injected C^{14} labeled estrone and DES into medaka eggs shortly after fertilization and reported 100% and 90% sex reversal, respectively. Labeled C^{14} estrone and DES were fed to larval medaka during the critical period of gonadal differentiation with 84% and 100% sex inversion resulting (Hishida, 1965). On recovery of carbon isotope, Hishida (1965) suggested that active accumulation of estrogens had possibly occurred in the differentiating gonad of larvae.

More currently, Kawamoto (1973) treated medaka fry per os after hatching with 17B-estradiol and reported that several spermatogonia developed into oocytes in 8.0 mm fry.

Turner and Bagnara (1971) pointed to three important principles illustrated from estrogen studies with the medaka: (1) early gonads, as well as the accessory system, have the ability to differentiate in a direction opposite to their own genetic constitution; (2) early germ cells are bipotential and in spite of their own genetic constitution can become functional testes or ovaries; and (3) a critical period in development exists during which sex hormones are effective.

Yamamoto and Kajishima (1968) successfully induced sex reversal in male Carassius auratus (goldfish) by administering estrone per os to fry daily for 2 mo. A (YY) male was detected from mating an estrone induced (XY) female and normal (XY) male, confirming functional sex

reversal in the (XY) genotype and male heterogamety (Yamamoto, 1975). Okada (1964) reported that spermatogonia in male protogynous hermaphroditic Halichoeres poecilopterus (wrasse) no longer responded to estrogen treatment after natural sex reversal occurred, while androgen administration accelerated the process of masculinization. In Ctenopharyngodon idella, Kawamoto (1950) reported ovarian growth was stimulated by oral administration of estrone.

Use of estrogens in cichlids

Eckstein and Spira (1965) set precedence in sex hormone research with Tilapia spp when they attempted to induce sterilization in T. aurea by maintaining young fry in water containing stilbestrol (DES) or stilbestrol diphosphate at various concentrations for 5 to 6 wk. They reported complete destruction in gonads of fry treated for 3 to 4 wk at the time of gonadal differentiation. In a concurrent gonadal differentiation study, they suggested at 7-8 weeks of age, with a body length of 18-22 mm, gonadal differentiation takes place and that the ovary differentiates a week to 10 days before the testis. Al-Daham (1970) evaluated DES as an agent to control reproduction in T. aurea by treating both juvenile and adult groups. There was no effect on adults (35 to 62 g) held for 10 days in water treated at 1.0 mg per liter. Groups of fry 17-31 mm were kept for 6 and 12 day periods in DES-treated water. No sex reversal was evident; high mortalities occurred at greater concentrations and no

inhibition of gonad growth was obvious at intermediate concentrations tested.

In Haplochromis desfontainesii, a seemingly permanent feminization was experienced when sexually undifferentiated fry were sustained in water treated with estradiol at 125 mg per liter throughout their completion of gonadal differentiation (Reinboth, 1972b).

Hackmann and Reinboth (1974) obtained sex reversal in juvenile Hemihaplochromis multicolor in groups between day 11.5 and 16 after spawning, when held in water with 0.25 mg estradiol butyryl acetate per liter for only 48 h. They also reported the paradoxical results of feminizing effects from androgen treatments, as did Hackmann (1974) in Tilapia heudeloti, T. mossambica and Cichlasoma biocellatum.

By treating T. mossambica from 6 to 25 days of age with 50 ug ethinylestradiol/g diet, Nakamura and Takahashi (1973) achieved 100% sex inversion. Treatment covered the sexually indifferent stage and proceeded through the completion of sex differentiation stage. They noted hermaphroditic gonads resulted when treatment extended only partially into the two stages while no modification occurred when treatment lasted only during either one of the two stages. In the same species Becker (1969, cited by Reinboth, 1970) attempted to induce sex reversal through incomplete gonadectomy with negative results, while Macropodus opercularis and Betta splendens (Becker et al., 1975) regenerated a heterotypic gonad after this operation.

Recently, Sanico (1975) obtained no significant alteration of sex ratio when 7 - 11 mm T. aurea fry were fed estrone at 100 ug/g diet for 22 days. Similar results were realized in T. mossambica of equal size when given an equal dosage of estrone for 39 days (Inland Fisheries Project, Phillipines, 1975). Using estrone at dosage of 200 ug/g diet, Guerrero, in preliminary studies, obtained one male in 65 fry treated perorally for 60 days (personal communication).

Common names for species cited in the preceeding discussion follow the American Fisheries Society (1970) and updated estrogen nomenclature is from Steeber (1968).

III. MATERIALS AND METHODS

Fry production

On 1 May 1975, 150 female and 75 male 1 year-old fish were selected as potential brooders and stocked in E-76, a 0.04 ha earthen pond located at the Auburn University Fisheries Research Station. Average total length and weight of females and males, were 18.0 cm, 110 g and 21.0 cm, 158 g, respectively. Seventy percent of the brood fish were overwintered in Auburn facilities (Avault et al., 1968) and the remainder in a warm-water spring (average 22°C) at Gore's Fish Farm, Lumber City, Georgia. Only a few (4) of the brooders were known to have spawned previously.

The fry production pond received 20-20-8 fertilization at 45 kg/ha, beginning 15 April, at biweekly intervals to provide natural food organisms and more opaqueness or color to water to encourage spawning activity. A mixture of diesel fuel and gasoline in ratio of 4:1 in quantity sufficient to create an "oil slick" over the entire pond area was used at weekly intervals, from 9 to 25 May, to eliminate air-breathing aquatic organisms. A daily ration of No. 5 Purina Trout Chow was provided at 3% of body weight from stocking to termination of fry collection dates.

Collection and selection of fry

Fry were collected from 23 to 25 May with a 4.0 m seine lined with 1.0 mm^2 mesh nylon net. Most collecting was conducted from 0700 to 0900 hr.

Harvested fry were transported to indoor 300 liter fiberglass troughs to select fry of the desired size (8.0 to 11.0 mm total length). A small sample (100 to 150 fry) was first transferred to a small, shallow plastic container of a light (white or yellow) color and the smallest fry were first individually removed with a small dipnet, then increasingly larger fry were selected to a size approaching 11.0 mm. After 50 presumably acceptable fry were selected, they were reviewed again by measuring the largest fry to the nearest millimeter. If largest fry were greater than 11.0 mm, they were replaced. All tanks were sequentially stocked with lots of 50 fry, then in 50 fry increments until the required number was reached. Deformed or atypically behaving fry were not used.

Preparation of treatment diet

The three naturally occurring estrogens evaluated were estriol, estrone and 17B-estradiol. Table 1 summarizes some of the physical properties of the phenolic steroids (Stecher, 1968) which were purchased from Sigma Chemical Company, St. Louis, Missouri.

Table 1. Physical Properties of the Three Estrogens Evaluated

Estrogen	Molecular weight	Melting point (°C)	Solubility	
			water	Etoh
Estriol	288.37	282	insoluble	freely
Estrone	270.36	254.5-256	slight	freely
17B-Estradiol	272.37	173-179	insoluble	freely

Solutions containing 30 ug/ml (30 ppm), 60 ug/ml (60 ppm) and 120 ug/ml (120 ppm) for each of the three chemicals were prepared using 95% ethanol. A mechanical agitator was used to expedite dissolution of the powdered chemicals.

Pelleted Purina Trout Chow was passed through a 1.0 mm^2 screen and ground in a Wiley mill. A given amount of the ground feed (50 g) was mixed with a numerically equal volume (50 ml) of each prepared estrogen-alcohol solution in a 500 ml beaker using a glass stirring rod. The air-exposed resultant mixtures were placed under an exhaust hood for 24 hr, then oven-dried at 30° C overnight to entirely evaporate the alcohol. Components were added to each 100 g of medicated feed to enhance the palatability, nutritive value and stability (Table 2).

Table 2. Supplementary Components in Treatment Diets

Ingredient	Quantity (g)
Terramycin	0.1
uncoated ascorbic acid	0.04
vitamin premix	0.47
cod liver oil	5.0
ethoxyquin (66%)	0.03

After solvent evaporation, the treated feed was remixed with a mortar and pestle and the supplementary components added and mixed separately with a glass stirring rod. Following incorporation of all ingredients the diet was again mixed to assure a homogeneous feed. The control diet was prepared as the treated diet with the exception of estrogen addition. The feeds were stored in airtight plastic bags and kept under refrigeration.

Experimental design

Ten groups containing 8.0 to 11.0 mm gonadally undifferentiated (Eckstein and Spira. 1965) S. aureus fry were stocked in separate treatment tanks. Each of the three estrogens was evaluated as to sex reversal potential in genotypic males at dosage levels of 30 ug/ , 60 ug/ and 120 ug/g diet. Each estrogen at indicated concentrations was administered for time regimes of 3 and 5 wk. Controls were used for both time periods. On termination of 3-wk treatment, approximately 50% of the fry in each of

the ten treatments was removed and stocked in outdoor concrete tanks. The remaining fry continued receiving their prepared diets for an additional 2 wk (5 total weeks), after which, they were likewise reared outdoors.

Treatment procedures

Groups of 350 fry each were stocked in four 151 liter (244 cm x 30 cm x 21 cm) and six 132 liter (210 cm x 30 cm x 21 cm) stainless steel tanks. Nine tanks were randomly assigned a particular estrogen treatment and one designated the control or untreated. To encourage feeding activity, fry were contained in a restricted area of 25 liters (72 cm x 30 cm x 21 cm) beneath the water inflow by a 1.0 mm^2 nylon mesh screen barrier (Figure 1). A similar nylon mesh was located over the drain standpipe to prevent fry escapement.

Each tank was equipped with an adjustable, continuous flow water supply which passed through an activated charcoal-oyster shell-gravel filter before entering the treatment tank. Free and combined available chlorine was checked colorimetrically weekly with orthotolidine reagent (Swingle, 1969). A water flow of 1.5 liters/min was maintained which completely replenished the water each 100 and 88 min in 151 and 132 liter tanks, respectively. Water temperature was maintained between 22.5° and 23.5°C during treatment period.

Fluorescent overhead lights in the treatment laboratory were covered with aluminum foil to avoid bright light reflections on the inside walls of the treatment containers.

Fry were acclimated to laboratory environment and conditioned with an untreated artifical diet for 2 days before initiating the experiment. Fry were fed 12% of estimated body weight for the first treatment week and 10% thereafter until treatments were completed. The daily ration was adjusted weekly by weights obtained from recovered deaths which were checked daily. The per diem ration was divided into five equal portions and presented at 0800, 1100, 1400, 1700 and 2000 hr, 7 days per week for duration of treatment.

Potassium permanganate was used as a weekly flush treatment of 3-4 ppm to serve as a prophylatic measure in combating water-borne pathogens. Treatment tanks were cleaned weekly to remove excess feed and extraneous debris.

Assessment of early sexual dimorphism

A bimodality in length within individual treatments was observed during the 3-wk treatment stocking. To determine if sex-related growth difference was evident at this age, as was reported in older individuals (Yashouv, 1958; Shell, 1967), fry were segregated into two groups. Fry in six 3 wk treatment groups were divided into small and large size categories using the approximate mean length of the entire group as the delimiting value. These two size groups in common treatments were stocked in separate outdoor tanks for growth. Upon harvest, the sex ratios in the two length categories from the six treatments were determined and analyzed statistically to assess possible significant differences (early sexual growth dimorphism).

Post-treatment growth in outdoor tanks

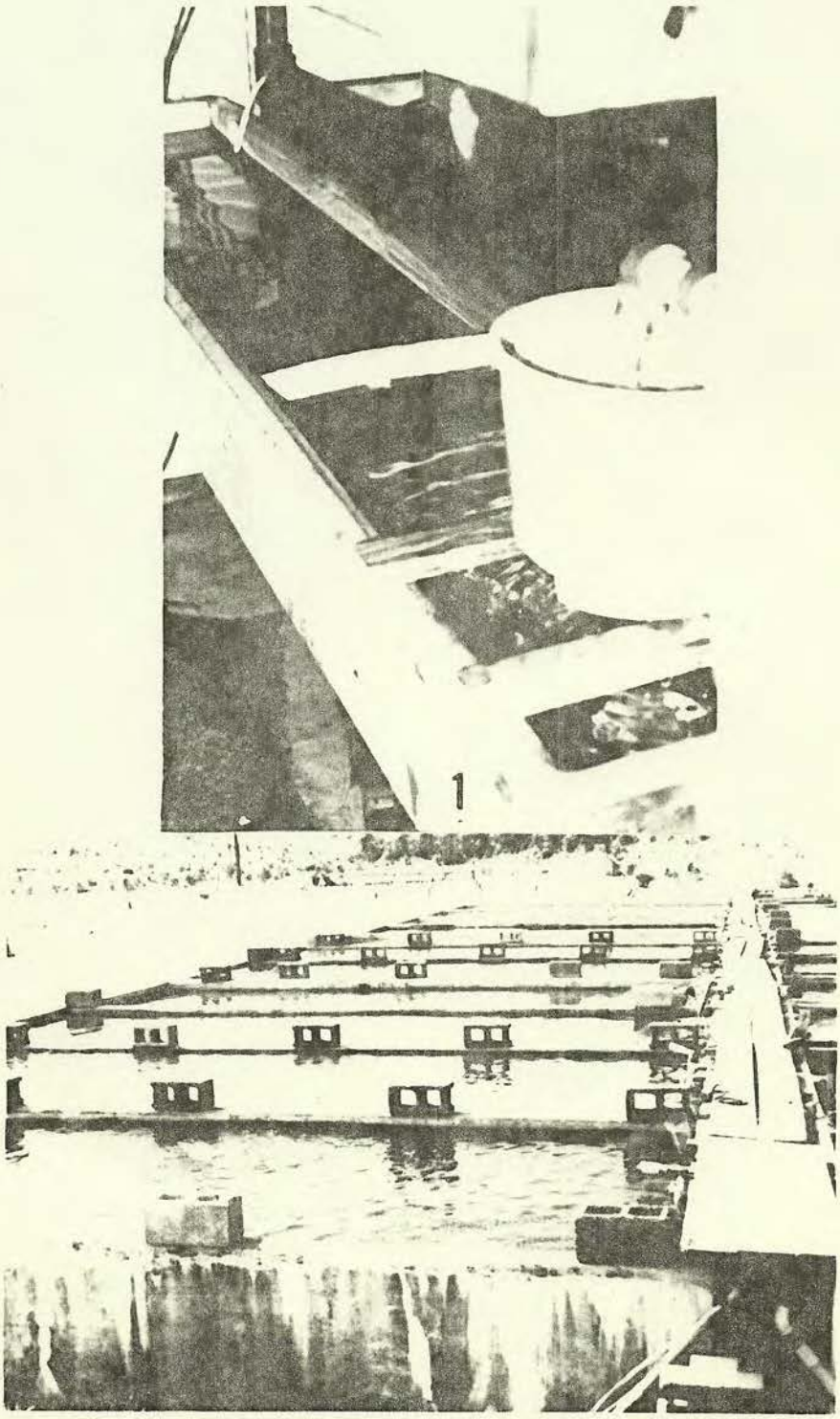
On completion of administration of estrogens, fry were stocked in 0.002 ha outdoor concrete tanks (8.0 m x 3.0 m x 0.8 m) as described by Shell (1966) and illustrated in Figure 2. Twenty-one of the 26 tanks were supplied with forced air bubbled through a perforated PVC pipe located on the tank bottom. Water supply was from a pumped well.

Prior to stocking, all tanks were fertilized with inorganic fertilizer to stimulate plankton proliferation for fry food and to provide protection from fish-eating birds. Tanks were also treated with diesel fuel-gasoline mixture as previously mentioned.

Tanks were initially stocked with 86 to 203 fry each (43,000 to 101,500 fry/ha) with a mean value of 128 fry (64,250 fry/ha). All tanks were fed the same ration, 3% body weight based on mean stocking figure adjusted biweekly. Because of lack of replications, questionability of tanks as useful experimental units (Shell, 1966) and differing growth days, no valid comparisons of growth data between different treatment groups were considered. Fish were fed with the objective of growth to an externally sexable size (10-13 cm) by September or October. As fish gained weight, the ration was not maintained at 3% due to development of dense phytoplankton blooms which created oxygen deficits. Feeding was discontinued when low oxygen tensions were suspected and submersible electric pumps used to maintain desirable dissolved oxygen levels (greater

Figure 1. Indoor treatment tank with screened barrier for fry confinement during estrogen administration.

Figure 2. Outdoor 0.002 ha concrete tanks used for post-treatment growth.



than 5 ppm). Commercially prepared Purina Trout Chow No. 4 was used in initial feeding stage then No.5 as fish approached 10 cm total length.

When possible, observed mortalities were retrieved and the gonadal tissue removed and examined using an aceto-carmine squash technique (Guerrero and Shelton, 1974).

On harvest, tanks were completely drained and all recovered fish transferred to treatment laboratory for observation and sexing.

Evaluation of sex ratios

The phenotypic sex was determined in all fish recovered from growth tanks, first by external examination of the genital papilla, with final confirmation by gross observation of gonads, or if required, by light microscopy. The difference in the sexual dimorphic genitalia in Tilapia spp. is described by Lowe (1955a) and Bard et al. (1974). To sex some stunted or atypical-appearing fish, gonads were examined by the technique employed by Guerrero and Shelton (1974).

Sex ratios in all treatments were compared as to signficant differences with expected values and variation between and within treatments, treatment dosage levels and treatment periods using the chi-square analysis for a contingency table and test for heterogeneity (Snedecor, 1955).

Histological studies

Samples of ovarian and testicular tissue from all experimental and control groups were fixed expeditiously on sacrifice of a specimen in either

10% formalin solution or Bouin's fixative. Fixed sections no larger than 5 mm^2 were later infiltrated with paraffin in a Lipshaw automatic tissue processor, then properly oriented and embedded in paraplast (mp, 56-57°C). The paraffin blocks containing tissue samples were sectioned with a rotary American Optical Microtome at a thickness of 8 to 10 microns.

The yellow color imparted in tissue from Bouin's fixative was removed by soaking microscopic slides with affixed tissue for 10 to 15 min in a 70% isopropyl alcohol solution saturated with lithium carbonate. The mounted sections were processed in a standard deparaffin, hydration and dehydration series with minor modification (isopropyl alcohol substituted for ethanol) (Humason, 1972). Tissue sections were stained with Harris hematoxylin, then counterstained with eosin. Coverslips were mounted on prepared microscopic slides with Coverbond.

Early ontological study

It was desirable to ascertain the approximate age and size of S. aureus when they hatch, absorb their embryological yolk sac and are able to consume artificial diets. This information would serve to estimate the stage of development and behavior of fry earlier selected for treatment.

On 5 March at 1400 hr, a 13.0 cm, 40 g female was observed orally incubating eggs, characterized by the obvious distension of gular region, which is illustrated by Shaw and Aronson (1954) in mouthbrooding T. macrocephala. Approximately 30 viable eggs were recovered by flushing water over the gills from behind the opercular flaps with a syringe.

Collected eggs were in the stage of early cell division (16-32) as described and illustrated by Shaw and Aronson (1954) in T. macrocephala. The female had been held in 28°C water 2 wk prior to her discovery.

Eggs were artificially incubated with modification of the system employed by Rothbard and Pruginin (1975). Incubation was at 25°C in a 500 ml Erlenmyer flask attached to a mechanical shaker for gentle water agitation and air diffusion. Water was replaced twice daily during the incubation period.

On hatching, yolk sac fry were maintained on hard-boiled egg yolk, and later on finely ground Purina Trout Chow. Fry were held in a 500 ml Erlenmyer flask with air supplied via an airstone and water at 25°C, renewed daily. Beginning on 11 March, a sample of two fry were preserved in 5% formalin solution approximately every 2 days until 1 April. Besides behavioral and feeding observations, length and weight data were recorded on samplings.

IV. RESULTS AND DISCUSSION

Brood spawning and fry procurement

Three days after stocking (4 May), several males had excavated nests in the shoal areas of the spawning pond. On 6 May with diurnal water temperature of 20-27°C, the optimum spawning areas had been partitioned into territories, each guarded by a male. With 23-29°C diurnal water temperature on 17 May, three of five females seined and examined were either carrying eggs or yolk-sac fry in their buccal cavities. Several days later, schools of fry were observed along the pond margin, and from 23-25 May, 3550 fry less than 11.0 mm were collected. Spawning was believed to have been initiated during the second week in May which agrees with data from McBay (1961), Fishelson (1966) and Dadzie (1970).

The brood fish density of 5625/ha in sex ratio of 2♀ : 1♂ was effective for adequate fry production. Lovshin et al. (1974) reported using 355 m^2 earthen and 36 m^2 concrete-sided ponds as spawning facilities for production of tilapia monosex hybrid fry. High fry production was obtained stocking 231 to 845 females/ha with 3♀ : 1-2♂. Lovshin and DaSilva (1975) also described two types of experimental spawning ponds designed to improve continual fry production by eliminating delay in conventional

brood fish restocking after fry collection. Problems encountered in fry collection from earthen ponds precipitated the evaluation of 2 x 1 x 1 m nylon net enclosures (hapas) for fry production (Inland Fisheries Project, Philippines, 1975). They reported 1920-5363, 8-11 mm fry were produced in 1 mo by stocking 12-21 T. mossambica/m^2 in ratio of 2-5 ♀: 1 ♂. Uchida and King (1962) tested sex ratios of 2-6 ♀ : 1 ♂ in T. mossambica and obtained best mean monthly fry production of 60,250 when brood fish were stocked at 10.25/m^2 in ratio of 3 ♀: 1♂ . Later, Hida et al. (1962) reported higher mean monthly fry production when brood stock density was increased to 11.5/m^2 with same sex ratio. Sex ratios from 2♀: 1 ♂ (Bardach et al., 1972) to as high as 5 ♀: 1 ♂ (Rothbard and Pruginin, 1975) have been utilized in tilapia fry production ponds. A smaller proportion of males is used to reduce aggression in breeding areas and because one male can mate with more than one female even on the same day (Fishelson, 1966).

A new approach to tilapia fry production is a multi-purpose breeding hatchery, involving year-round tilapia spawning in aquaria (Mires, 1973). In the Southeastern United States, winter water temperatures are below tolerance levels for tilapia and special overwintering is required which usually is not conducive to continual spawning activity.

First evidence of reproduction after stocking age-I overwintered fish coincided with results reported by Yashouv (1958), McBay (1961) and Rothbard and Pruginin (1975). McBay (1961) described S. aureus prespawning and spawning behavior and activities, some of which were

observed, and found to be typical of mouthbrooding cichlids (Breder and Rosen, 1966). The experimental 8-11 mm fry selected were estimated to be 8 to 21 days old post-hatching.

From fertility data available on S. aureus (McBay, 1961; Dunseth, 1975), some 30,000 to 45,000 fry could potentially be produced from the 150, 18 cm females originally stocked on their first spawn. From observations of the many thousands of fry collected and discarded due to their unacceptable size (larger than 11.0 mm), this estimate of fry production for May is probably conservative. Fry collection should have been initiated 1 wk earlier to assure a higher proportion of desirably sized fry and minimize sorting labor.

Spawning is usually asynchronous in tropical areas (Hyder, 1970), but because most brood fish were overwintered under optimum gonad developing conditions (water temperature above 23°C, fed high protein diet, and extended photoperiod), the first spawn after pond stocking could have been synchronous. Gonadal development was monitored during the overwintering period from October to March and revealed a marked increase in mid-February with gonadosomatic indices reaching 2.25-4.3. This GSI indicates a ripe ovary state in closely related Tilapia leucosticta (Welcomme, 1967). Some spawning occurred in crowded overwintering tanks from 23 February to 2 March.

Treatment phase

Treatment system efficiency

The confinement or crowding of fry in treatment tanks possibly affected growth but it encouraged active feeding, permitted easy tank cleaning with minimal fry handling and resulted in low mortality. No significant difference in growth between confined (463 fry/m^2) vs. unconfined (159 fry/m^2) groups was noted in another study (Shelton, unpublished data). At the end of 3-wk treatment period the mean length of all treatment groups was 17.7 mm. Guerrero (1974) and Sanico (1975) reported superior overall mean lengths of 19.1 and 18.9 mm, respectively, after hormone treatment of S. aureus fry for an equivalent treatment period in the same tanks. The confined groups in this study were stocked at 1620 fry/m^2 compared to considerably lower stocking rates of 159/m^2 and 318/m^2 for Guerrero (1974) and Sanico (1975), respectively.

The lower growth may be due to crowding or other factors such as differences in initial lengths, feeding practices, type of sex hormone tested, handling of fish and rearing environment. Uchida and King (1962) reported decreased growth of T. mossambica fry with higher stocking densities in outdoor rearing tanks. The best stocking density of those evaluated was 162 fry/m^2 water surface. They also stated fry did not feed on prepared diets until 2 wk after outdoor stocking. This could be the result of food preference of natural organisms over artificial diets as mentioned by Yamamoto (1958), where he stressed the importance of indoor treatment with sex

hormones. Yamamoto and Kajishima (1968) pointed out that variations in feeding, water temperature and degree of crowding greatly affected growth of C. auratus (goldfish) fry in single containers in which striking differences developed after 2 mo.

Feeding rings have been used by other investigators (Clemens and Inslee, 1968; Guerrero, 1975) to condition fry to feeding. One feeding ring per tank was tried but problems with diet adhering to it prompted its removal; furthermore, it was not necessary with the crowding practice. The amount of ration and its five daily presentations supplied sufficient feed with some excess after each feeding. The uneaten feed was pushed to the base of screened barrier where it accumulated allowing easy removal while maintaining the majority of fry rearing area clean. Shell (1967) indicated that it is best to divide a daily ration into several portions as tilapia have a relatively small stomach capacity and if too much feed is added, fish become quickly gorged leaving feed on the bottom, where it is poorly utilized. In the present study, fry were observed to feed more actively at the surface and in the water column as feed descended, but bottom feeding was also noted.

Few mortalities were experienced during the treatment phase, although several fish were unaccounted for and thought to have passed around the barrier and escaped down the drain. Few fry were observed outside the confined area, and if encountered, were returned and the barrier modified. Clemens and Inslee (1968) reported high mortalities when treating T. mossambica fry in 1 - 5 gal aquaria due to extra cleaning but obtained

complete sex reversal with proper androgen dosage vs. incomplete sex inversion in fry held in 100-gal metal tanks given the same treatment concentration per os. Fewer deaths occurred in the larger tanks and incomplete reversal was speculated to be due to availability of natural food which caused fry to consume less medicated feed.

A filtering system kept free chlorine levels below 0.1 ppm which is well under the concentration (> 0.5 ppm) reported harmful to small tilapia (Eren and Langer, 1973). In the following discussions of treatments, the unit ug/g diet, is deleted for simplicity and brevity, e.g. estradiol 60 ug/g diet is estradiol 60, etc.

Recovery of treated fry

No significant known mortalities were experienced during the treatment period (0.7%), but 5.2% of total fry treated was not recovered (Table 3). The mean recovery for all experimental groups was 94.2% compared to 93.4% for the control. Seven of the nine treatments had equal or higher fry recoveries than the control; estrone 120 was the lowest with 84.3%, due entirely to lost fry. Forty-four percent of the total mortalities was recorded in the first week of treatment which was probably due to initial stocking and acclimatization. Thirty-two percent of total mortalities occurred in the control and 16% were mechanical, experienced during tank cleaning. Not considering unaccounted fry, the overall mean survival was 99.3%. Escapement of fry down the drainpipe is the most probable explanation to account for the lost fry.

TABLE 3

SUMMARY OF FRY RECOVERY DURING INDOOR TREATMENT PERIOD[1]

Treatment	Initial No.	No. Mortalities	No. Unaccounted	No. Recovered	% Recovery
Control	350	8	15	327	93.4
Estriol	350	0	13	337	96.3
Estriol 60	400[2]	2	23	375	93.7
Estriol 120	350	1	22	327	93.4
Estrone 30	350	4	6	340	97.1
Estrone 60	350	4	17	329	94.0
Estrone 120	350	0	55	295	84.3
Estradiol 30	350	0	9	341	97.4
Estradiol 60	350	3	22	325	92.9
Estradiol 120	350	3	2	345	98.6

[1]Combined recovery data for 3 and 5 week treatment periods

[2]Inadvertantly overstocked

Guerrero (1974) reported some problems with bacterial diseases during androgen treatment period which are thought to have been alleviated in this study by incorporation of Terramyacin into diet. No epizootics were experienced.

Growth of treated fry

Length-weight data after 3 wk of treatment revealed some differences between control and several experimental groups. The overall mean length and weight of treatment groups were 17.7 mm and 0.084 g, as compared to 18.0 mm and 0.088 g for the control group. There were no apparent relationships between growth and variations in estrogen levels or potencies, between and within the three estrogens tested. The same mean weight was observed after 3 wk for the control and estriol 60 which had an additional 50 fry (Table 4).

After 5 wk of treatment, the mean length and weight of the control were 26 mm and 0.313 g compared to the overall mean length and weight of experimental groups of 25.3 mm and 0.305 g (Table 5). Estriol 60, with the highest stocking density had grown less than other treatments by the end of 5 wk while it was ranked No. 3 after 3 wk of treatment. This suggests an increasing negative effect from crowding as fry grew. Estradiol 120, the treatment of most potent estrogenic strength (Hawk et al., 1954; Yamamoto, 1969) went from No. 4 in mean weight after 3 wk to No. 9 at end of 5 wk, but

TABLE 4

SUMMARY OF GROWTH DATA IN 3-WEEK TREATED FRY

Treatment	No. Fry Sampled	Mean Length (mm)	Length Range (mm)	Mean Wt. (g)
Control	171	18	13-21	0.088
Estriol	175	18	14-22	0.084
Estriol 60	175	18	13-24	0.088
Estriol 120	173	17	13-24	0.081
Estrone 30	173	18	13-23	0.089
Estrone 60	173	17	12-22	0.083
Estrone 120	175	18	14-24	0.091
Estradiol 30	173	18	12-26	0.083
Estradiol 60	173	17	13-23	0.079
Estradiol 120	174	18	12-25	0.086

TABLE 5

SUMMARY OF GROWTH DATA IN 5-WEEK TREATED FRY

Treatment	No. Sampled	Mean Length (mm)	Range Length (mm)	Mean Wt. (g)
Control	156	26	16-34	0.313
Estriol 30	165	25	16-35	0.302
Estriol 60	203	23	14-32	0.240
Estriol 120	154	27	19-38	0.323
Estrone 30	168	26	18-34	0.321
Estrone 60	163	26	17-34	0.310
Estrone 120	131	26	17-35	0.330
Estradiol 30	168	25	17-42	0.337
Estradiol 60	153	26	16-36	0.319
Estradiol 120	178	24	19-33	0.262

was the only treatment at highest dosage level (120) to show a significant downward shift in ranking with time.

Post-treatment growth phase

Culture system performance

The outdoor concrete tanks were somewhat difficult to manage, especially with the high feeding rates applied. Mean total alkalinity for the 26 tanks was 70 ppm, ranging from 50-95 ppm, while mean total hardness was 46 ppm and varied from 28-77 ppm. With the favorable alkalinity system and added nutrients from feed, phytoplankton thrived in all tanks creating high fluctuations in pH and occasional oxygen depletions. Feeding was suspended on several occasions in tanks where dissolved oxygen values dropped to less than 1.0 ppm during the critical early morning hours. In these instances, submersible pumps maintained oxygen concentrations from 5-6 ppm. Tanks lacking aeration experienced less oxygen problems than those aerated and had 88% mean fish recovery vs. 75% for aerated tanks. This difference is probably related to the transfer on 8-9 August of five treatments stocked in the unaerated K-series tanks to A-series tanks, also unaerated, but containing clean water. Water temperature at 15 cm depth in unaerated tanks was 1°C higher than that recorded in aerated tanks due to differences in water mixing from aeration. From initial stocking on 28 June to 29 October when the last treatment was harvested, minimum and maximum

water temperatures recorded at 40 cm depth were 14° and 36°C, respectively.

Shell (1966) demonstrated differences in response to fertilizer and subsequent fish growth between concrete tanks and earthen ponds and stated that in experiments in which the effect of a treatment depends on interaction of aquatic environment and treatment, the results obtained in concrete tanks should not be considered applicable to earthen ponds unless demonstrated by experimentation.

Recovery of treatment groups on harvest

The recovery of the 3-wk treatment groups at harvest ranged from 61.3% to 91.0% with an overall mean of 81.5% (Table 6). Of the proportion not recovered, 5% was recovered mortalities and 13.5% was unaccounted fish. Treatments which had lower recovery rates had either experienced severe oxygen depletions with subsequent mortalities or had many unaccounted fish.

The 5-wk treatment groups had a higher mean recovery of 87.2% ranging from 64.3% to 95.8% among the treatments (Table 7). Unharvested fish consisted of 4.3% recorded mortalities and 8.5% unaccounted. Again, lower recoveries were accredited to unaccounted fish rather than treatment effects. Several mortalities in estrone 60 - 5 wk treatment were attributed to an infection of Aeromonas hydrophila, diagnosed by the Southeastern Cooperative Fish Disease Project, which was successfully controlled by oral treatment with Furacin at 11 g/100 kg fish/day for 10 days.

TABLE 6

SUMMARY OF RECOVERY IN 3-WEEK TREATMENT GROUPS
STOCKED IN OUTDOOR 0.002 ha CONCRETE TANKS

Treatment	No. Stocked	No. Mortalities	No. Unaccounted	No. Harvested	% Recovery
Control	171	12	8	151	88.3
Estriol 30[1,n]	172	8	12	152	88.4
Estriol 60[n]	172	3	14	155	90.1
Estriol 120	173	24	28	121	69.9
Estrone 30	173	22	45	106	61.3
Estrone 60[n]	167	1	23	143	85.6
Estrone 120	164	2	42	120	73.2
Estradiol 30	173	5	26	142	82.1
Estradiol 60[n]	172	8	18	146	84.9
Estradiol 120[n]	167	1	14	152	91.0

[1]Underlined treatments were divided into small and large size groups after treatment and stocked in separate tanks for sex ratio comparisons.

[n]Designates unaerated tanks in non-underlined treatments and in one of two segregated size groups in underlined treatments.

TABLE 7

SUMMARY OF RECOVERY IN 5-WEEK TREATMENT GROUPS STOCKED IN OUTDOOR 0.002 ha CONCRETE TANKS

Treatment	No. Stocked	No. Mortalities	No. Unaccounted	No. Harvested	% Recovery
Control	156	6	7	143	91.7
Estriol 30	165	12	17	136	82.4
Estriol 60	203	4	7	192	94.6
Estriol 120	154	8	47	99	64.3
Estrone 30	167	13	20	134	80.2
Estrone 60	162	5	4	153	94.4
Estrone 120	131	1	25	105	80.1
Estradiol 30	168	3	4	161	95.8
Estradiol 60	153	6	8	139	90.8
Estradiol 120	178	13	0	165	92.7

The somewhat higher mean recovery (37.2% vs. 81.5%) in the 5-wk treatment groups, suggests no obvious negative effects on survival from additional 2 wk of treatment. The mean length of 3-wk treatment groups at stocking was 17.7 mm while 5-wk treatment groups was 25.3 mm, giving an initial survival advantage to larger stocked fry. No obvious recovery trends between and within 3-and 5-wk treatment groups, separated or combined, were evident.

Mortalities recorded were found floating on water surface. Possibly, deaths due to natural causes floated to surface during unattended hours then sank to the tank bottom or remained obscured in water. Fry predation by fish-eating birds and/or carnivorous aquatic invertebrates could have been involved in the loss of unaccounted fish. Many molted exoskeletons of predaceous dragonfly naiads were observed attached to tank walls. These Odonata larvae possess tracheal gills which enable them to survive oil treatments. Uchida and King (1962) reported cases of cannibalism among juvenile T. mossambica larger than 20 mm but this was not observed and was dismissed as a major cause of unrecovered mortalities. Few, if any, fish were suspected of escaping tanks on draining, as a fine-meshed wire screen was placed in drainpipe and another in drainage canal to trap possible escapees. Fish jumping to adjacent tanks or to the ground was also discounted as an 8-in high wall of chicken wire surrounded each tank.

The initial stocking rates were chosen with the objective of obtaining at least 100 fish from each treatment for sex ratio evaluations on completion

of growth phase. Only one of the 20 different experimental and control groups was below this goal (Table 7). Recoveries ranged from 99 to 165 fish per treatment, excluding estriol 60 - 5 wk treatment which initially was stocked with 50 extra fry. The cumulative recovery from initial treatment stocking to harvest from concrete tanks ranged from 62.9% to 90.6% among treatments.

Growth of treatment groups

Growth data on harvest of 3-and 5-wk treatment groups are presented in Tables 8 and 9, respectively. No growth comparisons between treatments were made due to lack of replications and differences in stocking rates, culture days and feeding practices between treatments. The number of growth days varied from 99 to 148 with a mean of 117 days for all treatment groups. The mean length, weight and growth days for 3-and 5-wk treatment groups were 145 mm, 51.1 g, 106 days and 128 mm, 34.7 g, 127 days respectively. The superior growth of 3-wk treatments cultured for fewer days was probably due to a longer rearing time under optimal conditions. Most of the 5-wk treatments were harvested in October when water temperatures were less suitable for weight gains. Avault and Shell (1968) obtained T. aurea of mean weight 19.4 g, when stocked at 100 per 0.002 ha concrete tank after 83 days. Sanico (1975) stocked 25 T. aurea fry in the same tanks, and reported that fish reached a mean weight of 22.7 g in 43 days during good growth temperatures and only 26.6 g after 74 growth days, many of which extended into the cooler month of October.

TABLE 8

SUMMARY OF GROWTH DATA FOR 3-WEEK TREATMENT GROUPS
STOCKED IN 0.002 ha CONCRETE TANKS

Treatment	Stocked		Recovered					
	No.	Total Wt (g)	No.	Total Wt (kg)	Mean Wt. (g)	Mean Length (mm)	Range Length (mm)	No. Growth Days
Control	171	15.0	151	6.84	45.3	139	99-161	117
Estriol 30[1]	172	14.5	152	9.76	64.2	153	113-179	97
Estriol 60	173	15.2	155	9.85	63.5	155	18-184	108
Estriol 120	173	14.0	121	5.89	41.2	143	101-181	112
Estrone 30	173	15.4	106	6.09	57.4	138	120-175	108
Estrone 60	167	13.9	143	6.28	43.9	143	93-176	109
Estrone 120	164	14.9	120	3.65	30.2	129	85-158	101
Estradiol 30	173	14.3	142	8.23	54.8	149	110-172	103
Estradiol 60	172	13.6	146	8.89	59.7	149	113-183	99
Estradiol 120	167	14.4	152	7.72	50.8	148	96-182	102

[1]Underlined treatments were divided into small and large size groups after treatment and stocked in separate tanks for sex ratio comparisons, all corresponding values are means of the two groups combined.

TABLE 9

SUMMARY OF GROWTH DATA FOR 5-WEEK TREATMENT GROUPS
STOCKED IN 0.002 ha CONCRETE TANKS

Treatment	Stocked		Recovered					
	No.	Total Wt (g)	No.	Total Wt (kg)	Mean Wt (g)	Mean Length (mm)	Range Length (mm)	No. Growth Days
Control	156	48.8	143	7.21	50.4	144	112-173	148
Estriol 30	165	49.9	136	4.10	30.2	125	96-162	109
Estriol 60	203	48.7	192	5.02	26.2	117	77-147	131
Estriol 120	154	49.7	99	3.75	37.9	129	93-150	109
Estrone 30	167	53.6	134	3.79	28.2	121	84-145	143
Estrone 60	162	50.3	153	5.28	34.5	131	93-162	145
Estrone 120	131	43.2	105	4.20	40.0	131	80-158	117
Estradiol 30	168	56.6	161	4.99	31.0	123	81-156	117
Estradiol 60	153	48.8	139	5.04	36.3	130	85-153	143
Estradiol 120	178	46.6	165	5.35	32.4	125	86-156	106

Avault and Shell (1968) reported T. aurea continued to accept artificial feeds at 13°C, while T. mossambica ceased feeding at approximately 15°C (Kelley, 1956). In the present study S. aureus was observed to feed at 14°C.

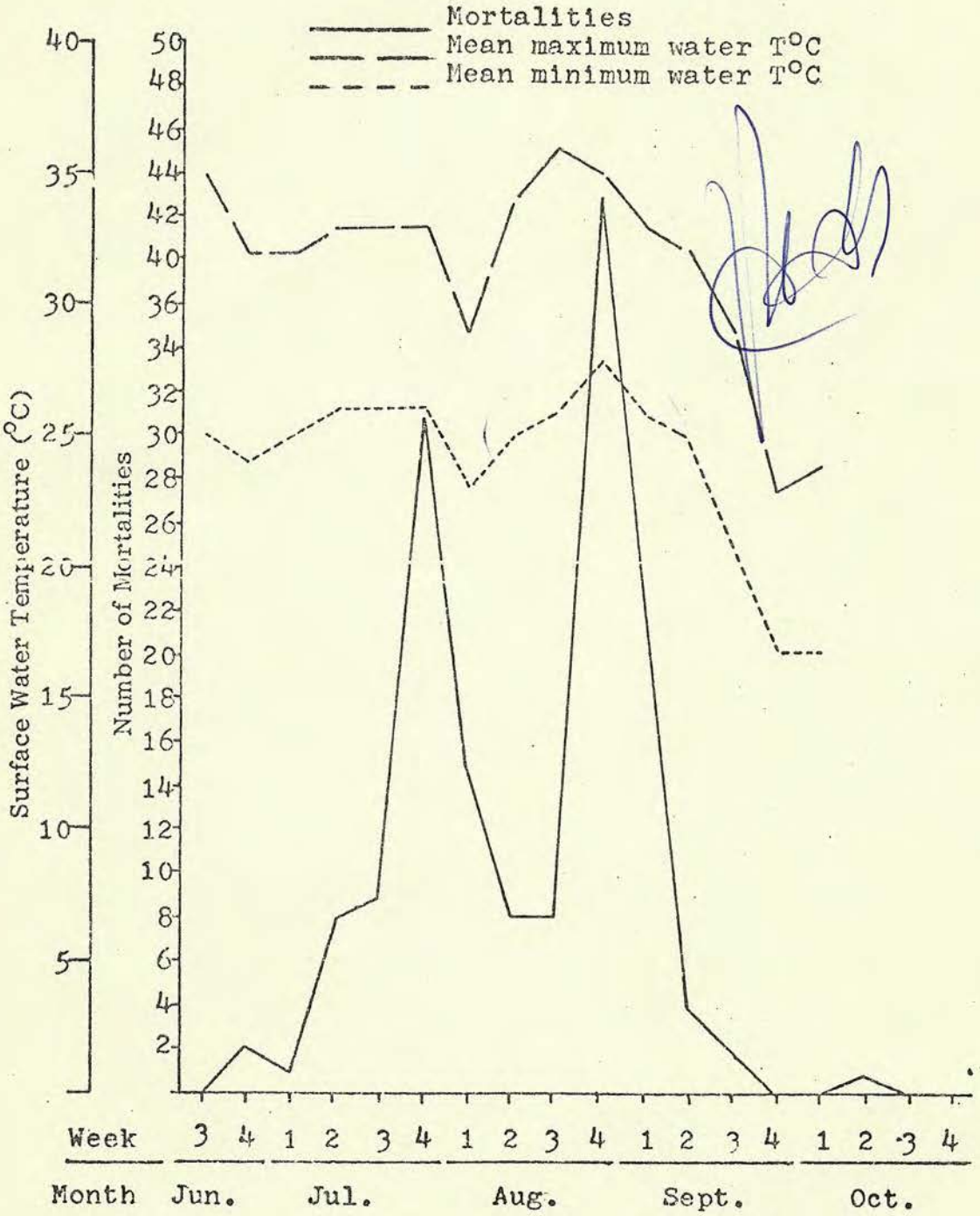
Evaluation of differential sexual mortality

Because mortalities could affect sex ratio of a treatment, an attempt was made to appraise possible sexual differential in recovered deaths. Ninety-seven fish were sexed; 40 were female and 57 were male. A t-test indicated no significant difference ($P \geq 0.05$) in mortality between sexes. Yamamoto (1953) mentioned that the sex ratio criterion is not always good to show evidence for sex reversal due to possible differential sexual mortality. Van Someren and Whitehead (1960) reported recovery of 75% males to 35% for female T. nigra when sexes were reared in separate ponds for 6 months. Survival of male T. mossambica was considerably lower than females in three different stocking densities evaluated in monosex culture in earthen ponds (Guerrero and Guerrero, 1975). Hickling (1959) reported a more similar mortality of 46% for males and 42% in females of age-I T. mossambica when grown in separate ponds.

Seasonal periodicity of post-treatment mortalities

Comparing recovered mortalities and minimum and maximum water temperatures in concrete tanks revealed similar trends (Figure 3). The greatest mortalities coincided with peak water temperatures while fewer

Figure 3. Seasonal Periodicity of Mortalities Recorded and Surface Water Temperatures in Concrete Tanks during Post-treatment Growth Phase



deaths were observed at lower water temperatures. A. hydrophila infection occurred during the first week in September about 14-16 days after the highest recorded temperatures. Meyer (1970) stated Aeromonas liquefaciens was the primary bacterial pathogen of farm-raised pond fish with its major occurrence in June, July and August, corresponding to the season of year with highest water temperatures. He further stated that bacterial infections occurred 10-14 days following the period when fish were subjected to stresses associated with low levels of dissolved oxygen. The high BOD values suspected in the concrete tanks with high temperatures and subsequent low oxygen values observed, appeared to be contributing factors in stressing fish, which in extreme cases, caused mortalities. This could also account for a few emaciated fish which were common to both experimental and control groups. Several investigators have reported a seasonal periodicity of fish pathogens and parasites related to water temperature (Snieszko, 1953; Rogers, 1969).

Observations of fish deformities and peculiarities

A significantly large proportion (23.8%) of fish harvested from growth tanks were deformed or possessed atypical features. Their growth was less than that for normal appearing fish, comparing overall mean lengths of 119 mm vs. 141 mm, respectively. The peculiarities involved indentation of dorsal fin, one or two degenerated eyes and eye sockets and a skin lesion extending from base of anal fin to vent area, usually found on

emaciated fish. The mean lengths of variously afflicted fish were 129 mm, 129 mm and 112 mm for dorsal identation, degenerative eye and lesioned fish, respectively. Of the total fish recovered, 2.3% had a depressed dorsal region, 7.1% possessed a degenerative eye condition and 14.4% experienced the anal fin lesion. The incidence of these features was equally distributed among experimental and control groups. The dorsal depression was observed on several brood fish which were overwintered in Georgia, suggesting a possible hereditary link if affected fry were their offspring. The degenerative eye formation as well as dorsal anomaly could be signs of inbreeding depression, nutritional deficiencies or parental heredity. Moav and Wohlfarth (1976) reported a high proportion of deformities in inbred Cyprinus carpio groups, i.e., over 60% of individuals had reduced dorsal fin, ranging from only a small reduction to complete absence. They further stated that these deformities are typical manifestations of inbreeding depression in common carp. Clemens and Inslee (1968) mentioned a high incidence of abnormalities including a small and poorly formed opercle, a deformed lower jaw and a foreshortened head in T. mossambica groups after androgen treatment. The atypical eyes appeared to be functional, but were considerably reduced in size. A scraping mark on skin of the pectoral girdle was often associated with the more serious anal fin lesion cases. Because these two areas are the lowest anterior-posterior ventral points on the body, it was suspected these abrasions with resultant lesions resulted from weak or stressed fish scraping the tank bottoms. The

emaciated condition of lesioned fish supported this theory. The bacterium, A. hydrophila, isolated from the lesion could have invaded exposed areas in weakened fish less resistant to the pathogen.

Appraisal of early sexual dimorphism

A bimodal size distribution during hormonal treatment suggested the possibility of an early sexual growth difference. Some treatments were divided into size groups at stocking. The sex ratio was determined at harvest. In the small size class, no resultant sex ratio deviated significantly ($P \geq 0.05$) from the expected 1:1 sex segregation (Table 10). In the larger size class ($\geq$17 mm), two groups had sex ratios that differed significantly ($P \leq 0.01$) from the expected, with males predominating (Table 10). A t test revealed no significant difference ($P \geq 0.05$) in pooled sex ratios between the two size categories, thus nullifying the hypothesis of possible sexual growth differences in S. aureus fry.

Fryer and Iles (1972) concluded that male growth superiority has a genetic basis in cichlids and is not associated solely with the reproductive process. Results indicated that sexual growth differences are manifested in juveniles at least larger than 26 mm. Pruginin and Shell (1962) reported T. aurea could be sexed when 13 g (9-10 cm) due to sexual distinctions in body thickness.

No differential sex mortality was suspected as recovery was greater than 90% in several groups. The two sex ratios that deviated significantly are thought to have been due to random chance as sample size was large.

TABLE 10

SUMMARY OF LENGTH-WEIGHT DATA AND SEX RATIOS IN 3-WEEK TREATMENT GROUPS SEGREGATED TO SIZE CLASSES TO ASSESS EARLY SEXUAL GROWTH DIMORPHISM

Treatment	Post-Treatment Size Classes: Small Length Range (mm)	Small Mean Wt. (g)	Large Length Range (mm)	Large Mean Wt (g)	Sex Ratio of Size Classes at Harvest: Small ♀ : ♂	Large ♀ : ♂
Estriol 30	14-17	0.063	18-22	0.107	1.03:1^{ns} (75)[1]	1:1.33^{ns} (77)
Estriol 60	13-17	0.064	18-24	0.113	1:1.03^{ns} (77)	1:1.89* (78)
Estriol 120	13-17	0.06	18-24	0.101	1.22:1^{ns} (60)	1.18:1^{ns} (61)
Estrone 30	13-17	0.063	18-23	0.115	1.3:1^{ns} (53)	1.21:1^{ns} (53)
Estradiol 30	12-17	0.059	18-26	0.106	1.24:1^{ns} (76)	1.06:1^{ns} (66)
Estradiol 60	13-17	0.055	18-23	0.102	1.44:1^{ns} (78)	1:2.23* (68)

^{ns}Not significantly different from the expected 1:1 sex ratio using chi-square test with Yate's correction ($P>0.05$)

* Significantly different from expected 1:1 sex ratio ($P<0.01$)

[1]( ) Number of fish sampled

Other tilapia species have produced aberrant sex ratios as Al-Daham (1970) reported ratio of 2.03 ♀ : 1♂ in control groups of T. hornorum. Sanico (1975) reported sex ratio of 2.29 ♀ : 1♂ in an untreated T. aurea group, but sample size was only 21 fish. Mires (1974) remarked that natural spawnings of pure strains of T. nilotica and T. shirana produced 80-100% males while T. aurea and T. vulcani yielded 45-70% males. He suggested that the departure from expected 1:1 sex ratio was due to some external factors, such as temperature or density or to differential mortality of female.

Sex ratios and sex reversal

The estrogens administered to presumably gonadally undifferentiated S. aureus fry did not appear to significantly alter their sex ratios. Only one of the nine treatments in 18 experimental groups evaluated proved to differ significantly ($P < 0.01$) from the expected 1:1 sex segregation (Table 11). Chi-square analyses for a contingency table revealed no differences in proportions of females within treatments tested for 3 wk, but highly significant differences ($P < 0.01$) in female distribution among the 5-wk treatments. The heterogeneity test showed no difference in the distribution and occurrence of females between the 3 and 5-wk treatment groups. No significant trends ($P \geq 0.05$) were observed in female proportions within different dosage levels for each estrogen individually, between estrogens and dosage levels tested, and between treatments and controls for both treatment periods. The absence of aberrant sex ratios indicates unsuccessful alteration of the primary sexual phenotype.

TABLE 11

SEX RATIOS[1] FOR TREATMENT GROUPS
RECEIVING MEDICATED DIETS FOR 3 AND 5 WEEKS

Treatment	Weeks of Treatment: 3 ♀:♂		5 ♀:♂	
Control	1.05: 1[ns]	(156)[2]	1.03: 1[ns]	(146)
Estriol 30	1: 1.21[ns]	(161)	1.04: 1[ns]	(145)
Estriol 60	1: 1.41[ns]	(157)	1.15: 1[ns]	(194)
Estriol 120	1.21: 1[ns]	(139)	1: 1.73*	(101)
Estrone 30	1.21: 1[ns]	(124)	1.20: 1[ns]	(143)
Estrone 60	1.09: 1[ns]	(144)	1.23: 1[ns]	(154)
Estrone 120	1: 1.02[ns]	(121)	1: 1[ns]	(106)
Estradiol 30	1.15: 1[ns]	(144)	1.19: 1[ns]	(164)
Estradiol 60	1: 1.17[ns]	(150)	1.24: 1[ns]	(141)
Estradiol 120	1: 1.12[ns]	(153)	1: 1[ns]	(170)

[ns] not significantly different from the expected 1: 1 sex ratio using chi-square test with Yate's correction ($P > 0.05$)

*significantly different from expected 1: 1 sex ratio ($P < 0.01$)

[1] sex determined by gonadal examination

[2] ( ) number of fish sampled

The only significantly different sex ratio deviated in the male direction, which is contrary to expected results following estrogen treatment. This treatment experienced the lowest recovery of 5-wk treatment groups (64.3%), but differential sexual mortality was evaluated in this study and found to be insignificant. Skewness in sex ratios of natural breeding intraspecific tilapia populations has been reported by other investigators (Al-Daham, 1970; Mires, 1974), but this has not been observed in the S. aureus stocks at the Auburn University Fisheries Research Station. Further replications could clarify this paradoxical observation which is thought to have occurred by chance and not through treatment effect.

While sex ratios indicated that no sex reversal had apparently been achieved, a comparison of genitalia and gonads revealed many individuals with female-like urogenital papillae (UGP) yet with developed testes. There are similarities of the UGP in a normal female and that found in an "atypical" male (Figure 4). This secondary sexual trait was manifested in only the estrogen treatments. The commonly found small urogenital pore in some males was widened so as to resemble an oviduct orifice and a gradation in the degree of similarity to the female UGP was noted. In many cases, males were first sexed externally as females, but internal sex verification revealed the presence of testes. This phenomenon focuses on the misleading results that can possibly be obtained from external sexing of hormone-treated fish, relying on secondary sex traits which are more readily modified than primary sex characters.

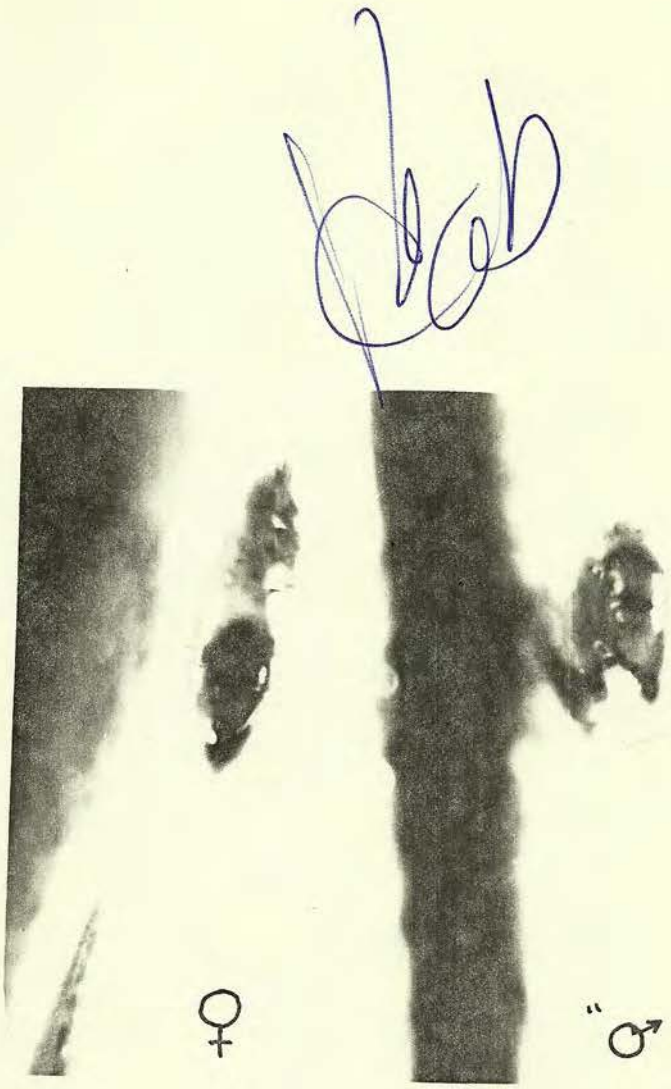


Figure 4. Similarity between Normal Female (♀) Urinogenital Papilla and that of an "Atypical" Male (♂).

No "atypical" males were observed in the control groups, reinforcing the theory that atypical papillae were estrogen induced. Of all recovered fish in experimental groups with testes, 34.2% possessed atypical genitalia. More than 45% of the males in treatments, estriol 120-5 wk, estrone 120-3 and 5 wk and estradiol 30 and 120-5 wk possessed this anomaly (Table 12). The trends within and between treatments and their durations are better observed in the graphic relationship between estrogen dosage levels and percentages of atypical males realized (Figure 5). Figure 6 reveals inconsistent results at the intermediate dosage level (60) in estradiol - 3 wk and 5 wk and estrone - 3 wk treatments. Exclusion of these data, shows a higher occurrence of "atypical" males with increasing estrogen concentrations as is expected. Further study of this relationship with replications could determine if erratic results are consistent and real or due to random chance in a single analysis.

A heterogeneity test ($P<0.01$) indicated the presence of a higher proportion of "atypical" males in the 5-wk vs. 3-wk treatments suggesting a more pronounced effect from the two additional treatment weeks. With the understanding that differences do exist between the two treatment regimes, Figure 6 depicts the more expected relationship between "atypical" male incidence and estrogen concentrations when data from both treatment periods are pooled. Combining data from the two treatment regimes revealed highly significant ($P< 0.01$) differences in occurrence of

TABLE 12

PERCENTAGES OF ATYPICAL MALES IN TREATMENT GROUPS

Treatment	Treatment Periods 3 Week	5 Week	Combined
Control	0 (73)[1]	0 (71)[1]	0
Estriol 30	28.4 (81)	22.2 (63)	25.7
Estriol 60	28.9 (90)	35.6 (90)	32.2
Estriol 120	32.7 (55)	45.2 (62)	39.3
Estrone 30	31.9 (47)	20.3 (59)	25.5
Estrone 60	15.9 (69)	35.3 (68)	25.5
Estrone 120	45.0 (60)	45.3 (53)	45.1
Estradiol 30	19.7 (66)	47.9 (73)	34.5
Estradiol 60	40.5 (79)	37.1 (62)	39.0
Estradiol 120	33.3 (81)	53.1 (81)	43.2

[1] ( ) sample size

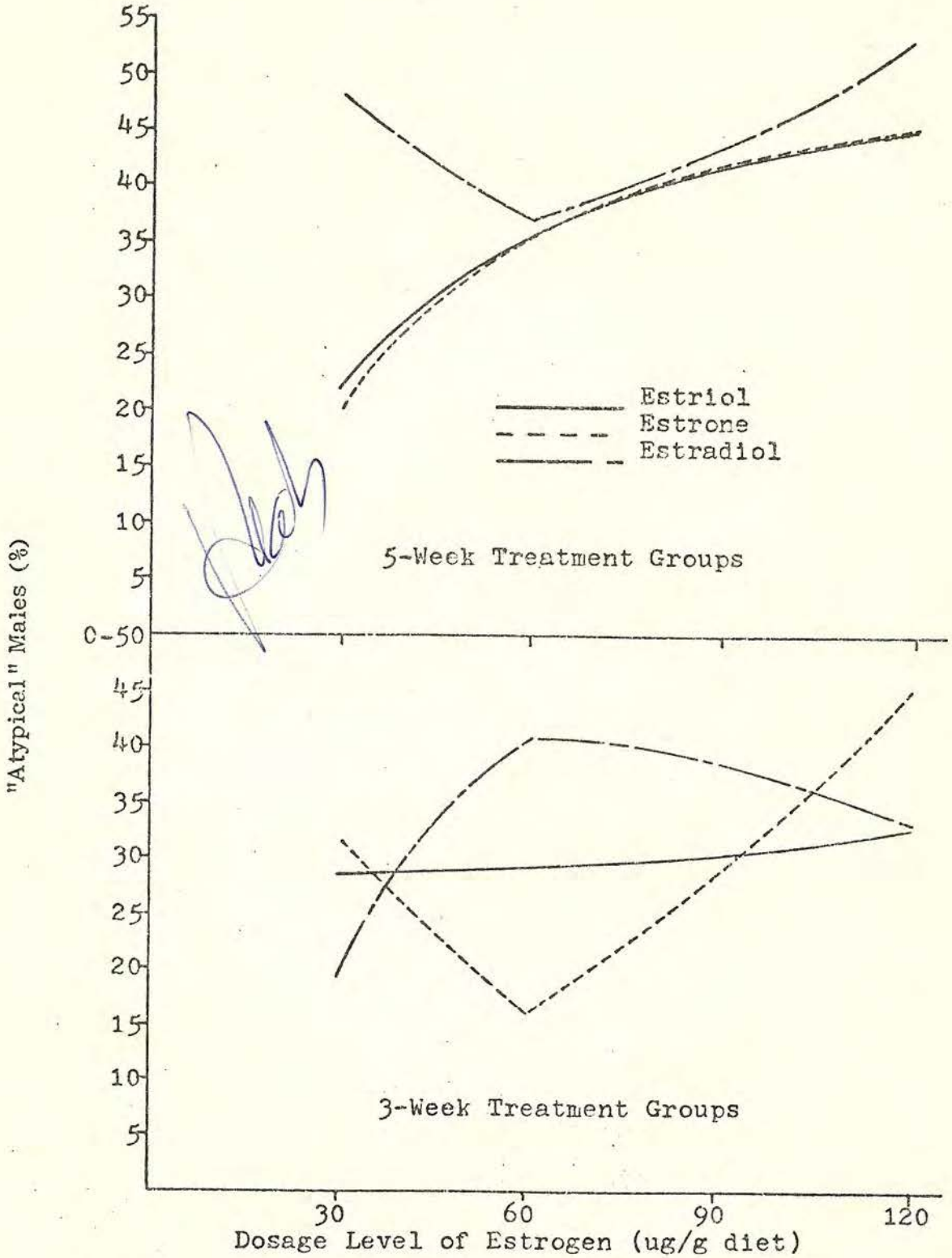


Figure 5. Relationship between Estrogen Dosage Levels and Percentages of "Atypical" Males in Experimental Groups in 3-and 5-Week Treatment Periods

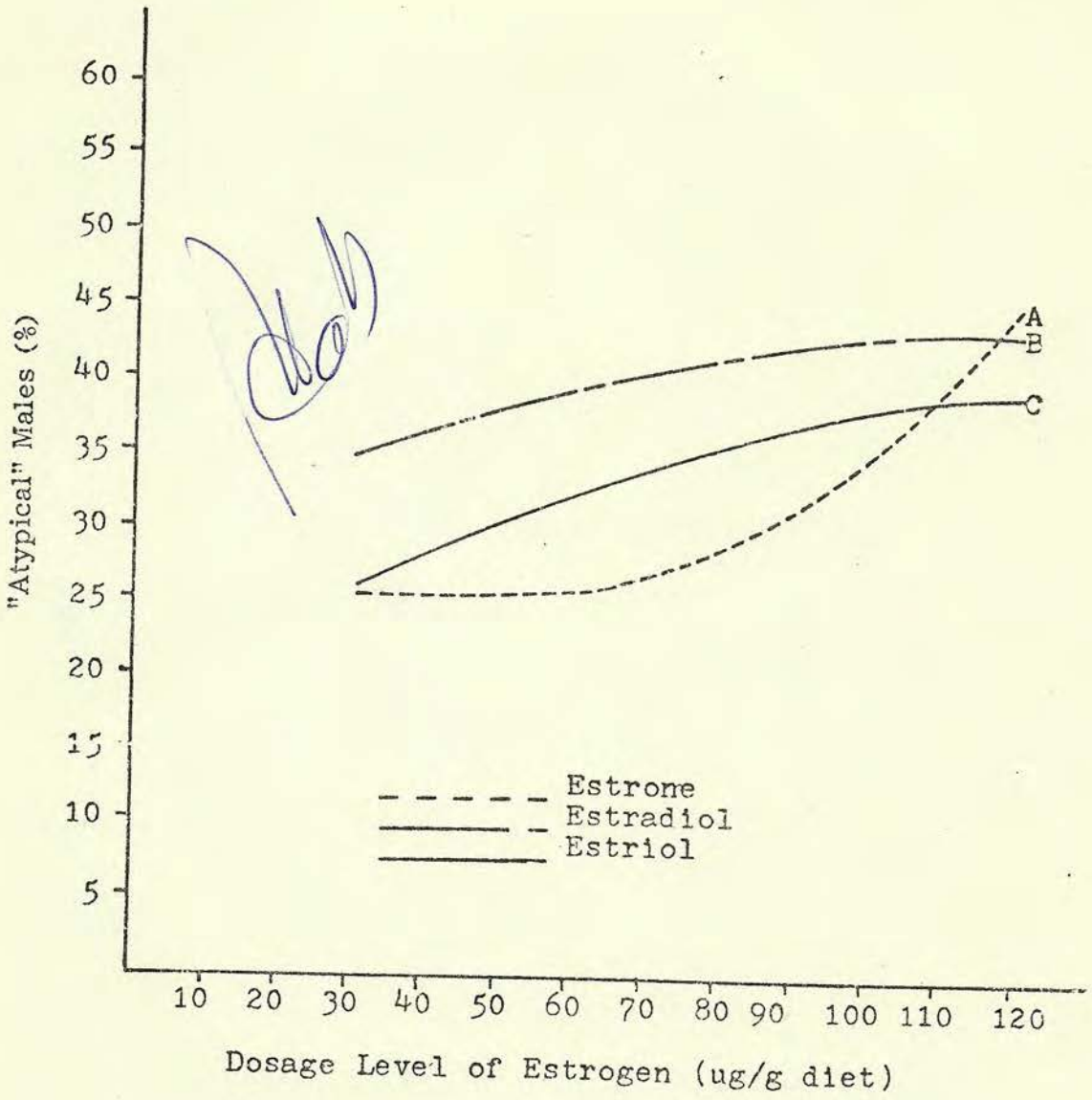


Figure 6. Relationship of Estrogen Concentration and "Atypical" Males Based on Pooled Data from Experimental Groups of Both Treatment Periods

"atypical" males between treatment groups. These observations demonstrate the need for treatment replications to make conclusive statements.

Yamamoto (1959a) demonstrated a linear relationship between estrogen concentrations and percentage of sex-reversed medaka males. Curves B and C in Figure 6 revealed a similar trend. Estrogenic potency in inducement of female-like genitalia in males, ranked 17B-estradiol, estriol and estrone in descending order of efficacy (Figure 6). Estrone surpassed both 17B-estradiol and estriol at 120 dosage level but was less at concentrations of 30 and 60. Yamamoto (1969) ranked 17B-estradiol, estrone and estriol in descending order of strength to induce sex reversal in male medakas. Fieser and Fieser (1959) mentioned that 17B-estradiol was more potent than estrone and estriol in subcutaneous treatment, while by per os administration, estriol was more active than estrone and 17B-estradiol in rats. This differential in efficacy with the mode of administration, could explain estriol's observed presumably higher potency over estrone in peroral treatment.

Chi-square analysis for a contingency table revealed highly significant ($P < 0.01$) differences in proportions of "atypical" males between 3-wk treatment groups. Most deviations were due to widely fluctuating intermediate dosage levels for estradiol and estrone (Figure 5). No significant differences in abundance of "atypical" males were noted between the three estrogens with pooled concentrations and between the varying dosage levels when equal concentrations were combined. The 5-wk treatments had highly significant differences ($P < 0.01$) in the proportion of "atypical"

males between the pooled concentrations for each estrogen, but no differences as regards the various concentrations when similar dosage levels were combined. The partial occurrence of female-like genitalia in males could be attributed to more active feeding and consumption of estrogens in affected fish. But, excluding the controls, the mean overall length for males with masculine UGP was 141 mm with standard deviation of 19.4 vs. 145 mm and standard deviation of 17.0 for those with feminine UGP.

Aronson (1948, 1951) provided evidence by gonadectomy and hormone treatment that the UGP in T. macrocephala is influenced directly by gonadal hormones. Furthermore, Bullough (1942) stated that the opening of the oviduct to the exterior in Phoxinus laevis is controlled by estrogens, while Ramaswami and Hasler (1955) reported that androgen treatment produced male characteristic breeding tubercles on female Hyborhynchus sp. Yamamoto and Suzuki (1955) in further experiments hypothesized that the medulla of UGP in medaka is manifested by both male and female gonadal hormones but its development is far more sensitive to estrogens.

Clemens and Inslee (1968) reported all T. mossambica with ovaries exhibited male-like genital papillae when treated with an androgen. Precocious manifestation of female-like UGP in all genotypes of medaka following estrogen treatment was observed by Yamamoto (1965). He also noted that non-reversed males lost the atypical UGP when they reached adulthood. It appears that either sex has the potential for development of secondary opposite sexual traits, but endogenous hormones apparently

overpowered the inherent hormonal secretions and inhibited or suppressed their effect, evidenced by formation of heterosexual UGP in some males.

Yamamoto (1965) stated that the medulla of the UGP in the medaka is insensitive to estrogens until fry reach a certain critical size. Hackmann and Reinboth (1974) also stated that the administration of exogenous hormones after the sensitive period for sex reversal, has only a partial, short-lasting effect on animal's sex and sometimes only secondary traits are influenced by experimental procedure. These reports suggest that the higher proportion of "atypical" males in the longer treatment duration groups reached a size sufficient to elicit a response to estrogens. Yamamoto (1965) further demonstrated that the minimum effective dosage level for inducing precocious manifestation of the UGP is lower than that for inducing reversal of gonadal sex differentiation in medaka. Kawamoto (1973) reached a similar conclusion when treating medaka with an androgen.

In the present study, it is believed that the consumption of sufficient amounts of estrogen during a critical stage in manifestation of UGP, induced gradations of feminization in some male UGP.

The explanation of failure to induce complete sex reversal in genetic male S. aureus by the treatments tested, rests in an insufficient estrogen strength, estrogen administration outside the sensitve period extending from the sexually indifferent stage through the stage of gonadal sex differentiation and/or detrimental inactivation of naturally occurring hormones in the gastrointestinal tract or liver. Guerrero (1974) stressed the importance

of androgen-treated 9-11 mm T. aurea fry to reach at least 19-22 mm for achievement of successful female sex reversal. The fact that the ovary differentiates 7 to 10 days before the testis in T. aurea (Eckstein and Spira, 1965) suggests that estrogen treatment should be continually administered to fry larger than 19-22 mm. Possibly, the testis is not completely differentiated until fry reach a larger size and if treatment is curtailed before completion of gonadal differentiation, no gonadal sexual modification is obtained. Guerrero (1974) reported no effect on gonadal alteration when T. aurea fry reached a mean length of 19.7 mm, while fry attaining 22 mm were completely sex reversed. Nakamura and Takahashi (1973) stated similar results when estrogen treatment failed to extend through the completion of the gonadal differentiation stage or indifferent stage in T. mossambica. Failure to extend treatment through this critical period could have resulted in unsuccessful sex reversal in 3-wk treatments, where their overall mean length was only 17.7 mm after treatment. On termination of the 5-wk treatment, the overall mean length in experimental groups had reached 25.3 mm. The likelihood also exists that this size is premature for completion of gonadal differentiation in S. aureus males. Guerrero's (personal communication) attainment of 98.5% females in a group of 8-11 mm T. mossambica treated with estrone at 200 ug/g diet for 60 days, suggests a treatment period longer than 5 wk is warranted. In this study, 17B-estradiol 120 has an equal or greater estrogenic strength than estrone 200 (Yamamoto, 1969).

Nakamura and Takahashi (1973) stated that gonadal sex differentiation occurs during 16 and 20 days post-hatching when T. mossambica fry are between 8-11 mm and that gonadal differentiation occurs during the same period for both sexes. Clemens and Inslee (1968) for the same species, reported gonadal differentiation occurring between 35-48 days post-hatching when fry were 15-30 mm. Nakamura and Takahashi's findings (1973) are thought to be more realistic; they demonstrated that ethinyl-estradiol administration to T. mossambica fry from 6-25 days post-hatching induced complete feminization in genetic males.

The estimated post-hatching age of the treated 8-11 mm S. aureus fry is about 7-21 days, while on the sixth day post-hatching, fry were 7 mm and observed to have absorbed their yolk sac. Eckstein and Spira (1965) estimated age of 9-11 mm T. aurea to be 28-35 days from the time of release from brooder's buccal cavity. Nakamura and Takahashi (1973) stated the age of 8-11 mm T. mossambica fry to be 16-20 days post-hatching. This would imply treating S. aureus fry from a size of 7 mm (6 day-old post-hatching) to some 15 mm (25 day-old post-hatching) with ethinylestradiol at 50 ug/g diet or an equally potent estrogenic concentration for complete feminization of males, if gonadal development is similar in both species.

Eckstein and Spira (1965) reported that the sex in gonads of T. aurea is first discriminated in 30 day-old 10-12 mm fry and that gonadal sex differentiation occurs when fry are 18-22 mm or 49-56 days old. Guerrero's results (1975) appear to support this as concerns androgen treatment to T.

aurea from 8-11 to 22 mm. Therefore, it is thought that sufficient estrogen treatment to S. aureus males from 8-11 mm to 24-26 mm should evoke a similar response (sex reversal).

An ineffective estrogen concentration could likewise have attributed to this study's lack of success. Using Table III from Yamamoto (1969), which presents comparative potencies of estrogens to induce 50% sex reversal in male medakas, Nakamura and Takahashi's 50 ug of ethinylestradiol, which proved successful to induce sex reversal in T. mossambica, is equivalent to 170 ug, 588 ug and 3823 ug for 17B-estradiol, estrone and estriol, respectively. The highest dosage level for any one of these estrogens was only 120 ug in this study.

In comparison, Guerrero (1975) in T. aurea and Clemens and Inslee (1968) in T. mossambica, achieved sex reversal in these species with an androgen concentration that similarly induced sex inversion in the medaka (Yamamoto, 1958). If the same relationship holds true for the males of both species treated with estrogens, then 13 ug 17B-estradiol (Yamamoto and Matusda, 1963) 40 ug estrone (Yamamoto, 1959) and 260 ug estriol (Yamamoto, 1965) per g diet should presumably produce complete feminization in S. aureus genotypic males. In this respect, an insufficient treatment period is suspected in producing unsuccessful sex reversal in this study, due to delayed differentiation of testes (Eckstein and Spira, 1965).

If UGP development is controlled by gonadal hormones which are elaborated from formed gonads, then completion of testes differentiation

before treatment's end is possible, because both normal and atypical UGP appeared in males. This implies subthreshold estrogen concentration as being the prime factor in yielding non-reversal results. This would also denote that the atypical males from 3-wk treatment groups were the largest, faster-growing fry which reached a size sufficient for termination of gonadal differentiation. Satoh (1974) noted that sex differentiation of germ and somatic cells preceded the differentiation of steroid-secreting cells in the medaka. In addition, Yamamoto (1965) stated that the precocious development of UGP occurs only in medaka fry larger than 14 mm, while sex hormone treatment to fry reaching only 12 mm (Yamamoto 1963, 1968) results in sex inversion.

In the case of medaka, treatment commenced immediately after hatching and lasted until fry reached 12-15 mm (Yamamoto, 1959, 1963). As observed in S. aureus, the medaka ovary differentiates earlier than the testes (Yamamoto, 1962; Onitake, 1972). But, in medaka, Satoh and Egami (1972) observed that morphological sex differentiation occurs at the time of hatching, not delayed until post-hatching as in tilapia (Eckstein and Spira, 1965; Nakamura and Takahashi, 1973). Kawamoto (1973) observed part of spermatogonia developing into oocytes in 8 mm medaka fry treated with 17B-estradiol at 20 ug/g diet.

Hackmann and Reinboth (1974) demonstrated the necessity to administer exogenous hormones between day 12-16 after egg laying to alter sex phenotype in H. multicolor with estrogens. They also showed that sex

hormone treatment earlier or later than this period produced no effect. Muller (1969, cited by Hackmann and Reinboth, 1974) stated that gonadal differentiation in this species becomes histologically visible around day 17 after oviposition. This was reported in T. mossambica (Nakamura and Takahashi, 1973). When hormone treatment partly covered the sensitive period, ovotestes condition resulted. The sensitive period appeared to correspond with a particular developmental stage in H. multicolor, when the development was retarded by decreasing temperature of environment, the specimen entered the critical period at a later time (16-18 days). A temperature decrease from 26.5°C to 23°C delayed gonadal differentiation approximately 2 days (Hackman and Reinboth, 1974).

Temperatures reported by various investigators in their experiments were 20± 1°C (Clemens and Inslee, 1968), 20± 2°C (Nakamura and Takahashi, 1973) and 23±0.5°C in this study. The temperature differences noted could affect extrapolation of results from one study to another. If Nakamura and Takahashi's reported gonadal development and differentiation hold true for S. aureus, the higher temperature in this study would suggest earlier gonadal development. Treatment would need to begin with yolk-sac fry of some 6 mm or 4 days old, post-hatching.

From this discussion, arguments can be made for either inadequate estrogen concentration or improper treatment period as the cause for ineffectual gonadal alteration. Because of the absence of intersexes in this study, it is hypothesized that the treatment period did not extend into both

the sexually indifferent and sex differentiation stages. If the testes in S. aureus differentiate 7-10 days after the ovary and successful androgen-induced males are not obtained until treated fry reach approximately 22 mm, then estrogen-induced females could only be obtained after treated fry reached over 25 mm. Guerrero (1974) mentioned that under artificial conditions the length of fry should be a more reliable criterion than age for determining when to start and end sex hormone treatment. The fry of 5-wk treatment groups were only 3.3 mm mean length larger than Guerrero's (1975) sex-reversed fry, yet they were 17 days older. With no sex reversal evidenced after 5 wk of treatment, with fry averaging 25 mm, a longer treatment period is suggested to enable fry to reach a larger size such that treatment lasts throughout the completion of the gonadal sex differentiation stage in males. The higher proportion of "atypical" males in the 5-wk treatment groups supports the extension of treatment regime. Many of the estrogen concentrations were too low, but 17β-estradiol 120 should elicit a response if administered for a sufficient period.

It appears that species difference, water temperature, and rearing system type can markedly affect fry growth and/or time and duration of gonadal differentiation. These differences among sex hormone treatment results with teleosts need to be considered for their proper interpretation and comparison.

Histological examination of gonads

The estrogens at the concentrations tested produced no evident effects on gonads of genetic females. The development of oogenesis and vitellogenesis was not obviously inhibited nor accelerated when comparing ovaries from control and experimental groups (Figures 7 and 8). A succession of oocyte sizes common to fish with a long, continous spawning period (as in tilapia) occurred in treatment and control groups (Figure 7).

The possibility of ovotestes was considered but none were found. However, the existence of oocyte-like elements were sparsely and evenly distributed through the periphery of testicular tissue, but were found equally in treated and untreated groups (Figure 9). These pseudo-oocytes which were usually embedded in the testis stroma and not within germinal cysts, resembled true oocytes as regards to cytoplasm, nucleus and a single nucleolus (Figures 10-12). Size and general morphology were also similar. Developing oocytes were surrounded by a theca (Figure 11) as were some testicular counterparts (Figure 10).

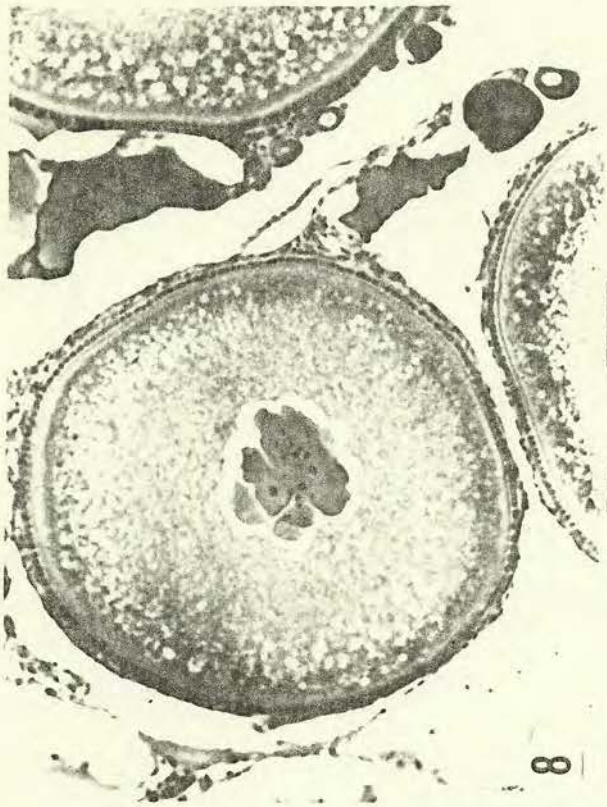
The testicular gonoduct system appeared to be unaltered by treatments (Figures 13-15). An obvious distinction in sperm collection and transportation system was noticed in cross-sections of posterior (Figure 13) and anterior (Figure 15) portions of the testes. Caudally, a Y-shaped lumen was located in the central stroma with a peripheral duct encompassing the entire gonad (Figure 13). This outer duct system is also plainly visible in longitudinal-sections (Figure 14). Nakamura and Takahashi (1973)

Figure 7. Ovary of fry treated with estrone 120 for 3 weeks. Note asynchronous development of oocytes. x35

Figure 8. Intraovarian oocyte undergoing vitellogenesis from 3 week control. x90

Figure 9. Pseudo-oocyte element (arrow) attached to wall of testi lobule in fish treated with estriol 120 for 3 weeks. x90

Figure 10. Oocyte-like structure (arrow) embedded in testicular matrix of fish treated with estradiol 120 for 5 weeks. x320.



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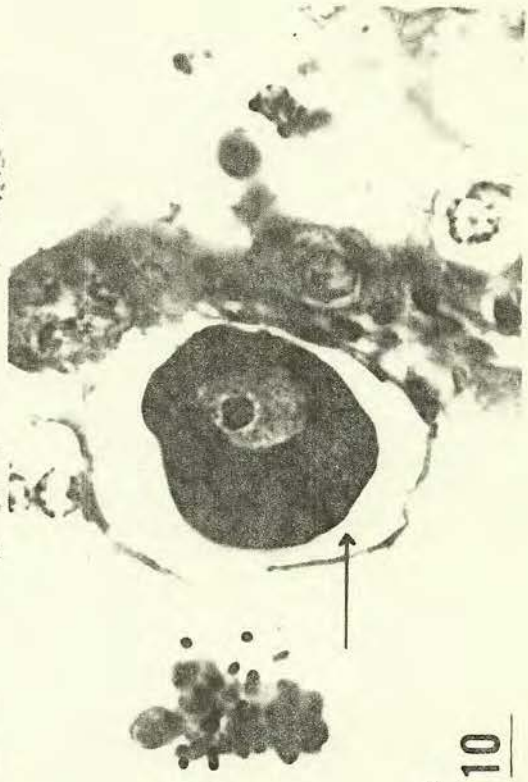

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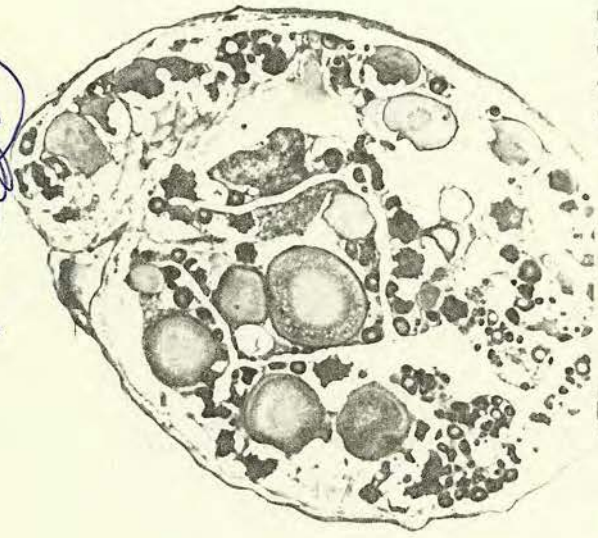

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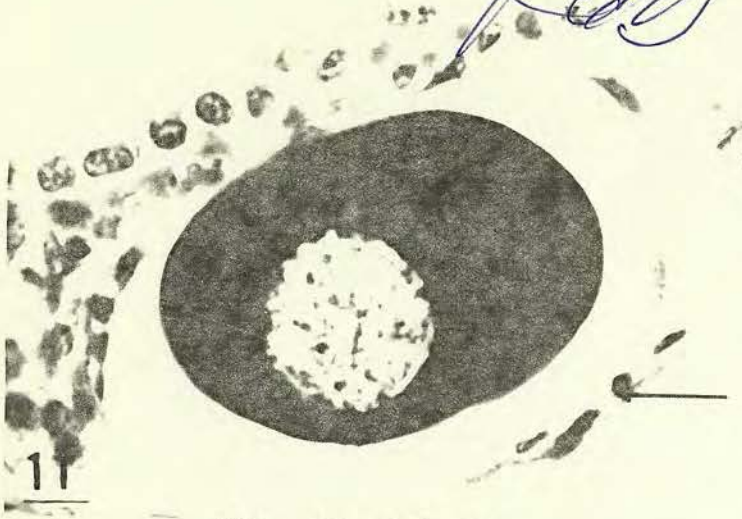
Figure 11. Developing intraovarian primary oocyte from fish treated with estrone 120 for 5 weeks. Note similarity of surrounding thecal layer (arrow) to that in Figure 10 associated with presumptive oocyte. x1145

Figure 12. Ubiquitous pseudo-oocyte (arrow) in testis of fish from 5 week control. x1145

Figure 13. Cross-section of posterior testis from fish treated with estrone 60 for 5 weeks. Note marginal gonoduct system (A) and central Y-shaped lumen (B). x35

Figure 14. Longitudinal-section of posterior testis from fish treated with estradiol 60 for 5 weeks. Note morphology of sperm collecting ducts (arrow). x35



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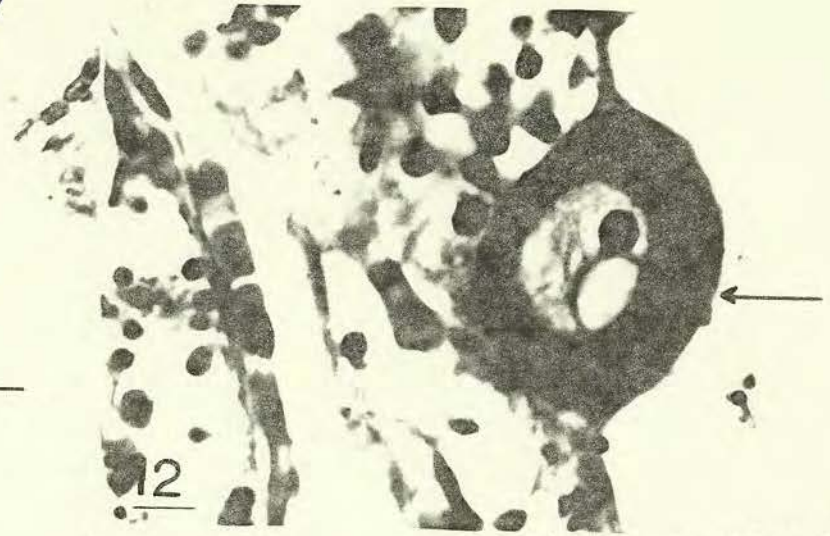

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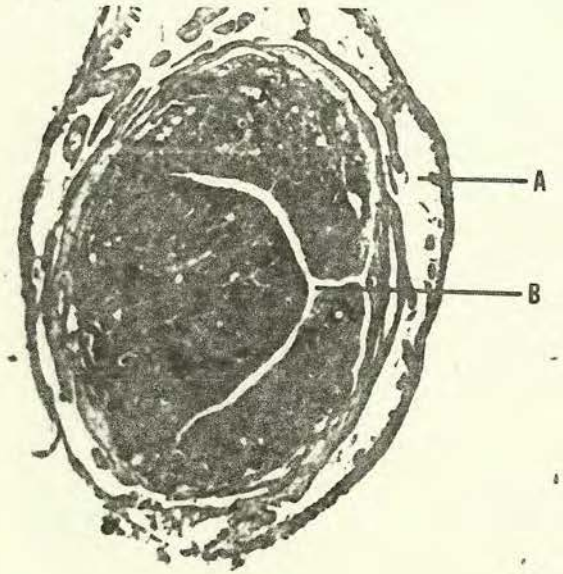

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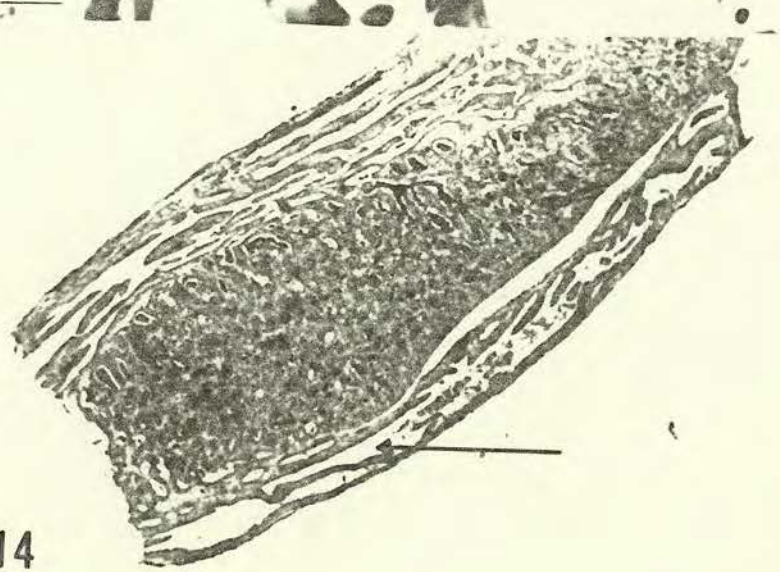

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illustrated a similar central lumen in stromal tissue in T. mossambica which they described as efferent ducts. Figure 15 shows the sperm ducts in caudal portion of testis which are similar to those exemplified by Hyder (1970) in T. nigra. Examination of columnar epithelial cells lining the sperm ducts (Hoar, 1957) also revealed no gross abnormalities (Figure 16). Chieffi (1959, cited by Miyamori, 1964) reported that estradiol benzoate had a feminizing action on the genital duct in elasmobranchs. Ethynylestradiol treatment to Lebistes reticulatus resulted in elimination of vas deferens due to a decrease in gonadal stroma cells (Miyamori, 1964).

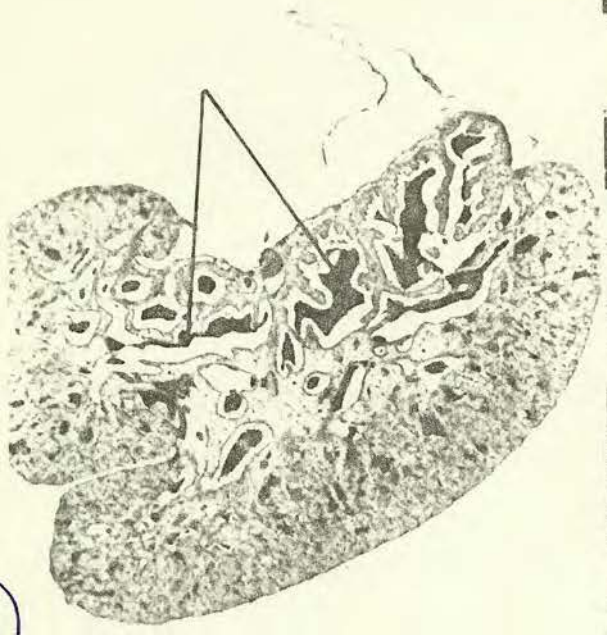
Spermatogenesis was neither inhibited nor suppressed by estrogen treatments as is commonly reported with little differences in germinal and stromal tissues between control and experimental groups. Figures 16 and 17 depict stages of spermatogenesis found in maturing tilapias (Hyder, 1969). Slight variations between individuals and treatments were thought attributed to differences in health, nutrition, growth days and time in optimum and suboptimum water temperatures, rather than treatment itself. Figure 17 illustrates normal spermatozoa formation in fish administered the most potent treatment. Intratesticular lobules are proliferating along with abundant germinal cysts. Individual cyst units are undergoing synchronous germ cell development and an accumulation of spermatozoa in lobule lumina are seen in Figure 18. Different stages of spermatogenesis are within the various cysts comprising this lobule from fish treated with estradiol 120 for

Figure 15. Cross-section of anterior testis from "atypical" male treated with estradiol 120 for 3 weeks. Compare gonoduct system (arrows) to that in Figure 13. x80

Figure 16. Cross-section of testis showing active spermatogenesis in maturing "atypical" male treated with estrone 60 for 5 weeks. x145

Figure 17. Cross-section of testis showing arrangement of lobules (A) with central lumen (B) and intralobular cysts (C) containing germ cells (D) in 172 mm fish treated with estradiol 120 for for 5 weeks. Compare predominance of cysts in early stages of spermatogenesis to those in Figure 16. x90

Figure 18. Cross-section of intratesticular lobule (A) with several cyst units (B) in different stages of synchronous spermatogenesis from fish treated with estradiol 120 for 5 weeks. x720



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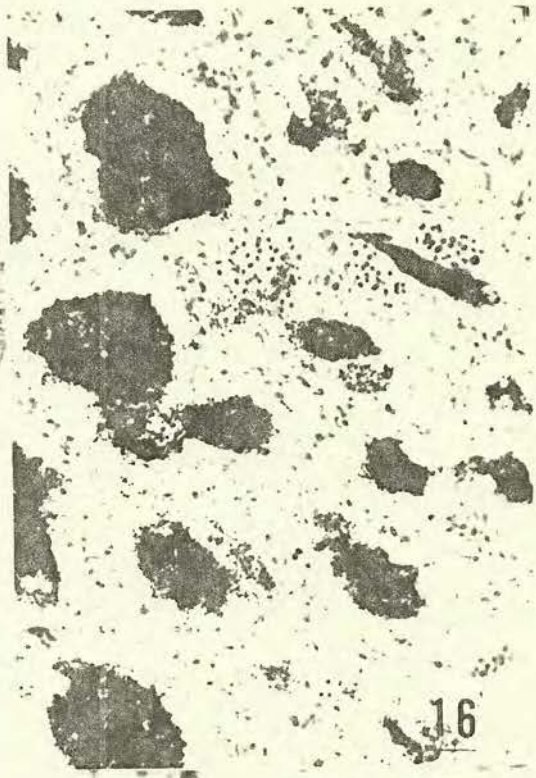

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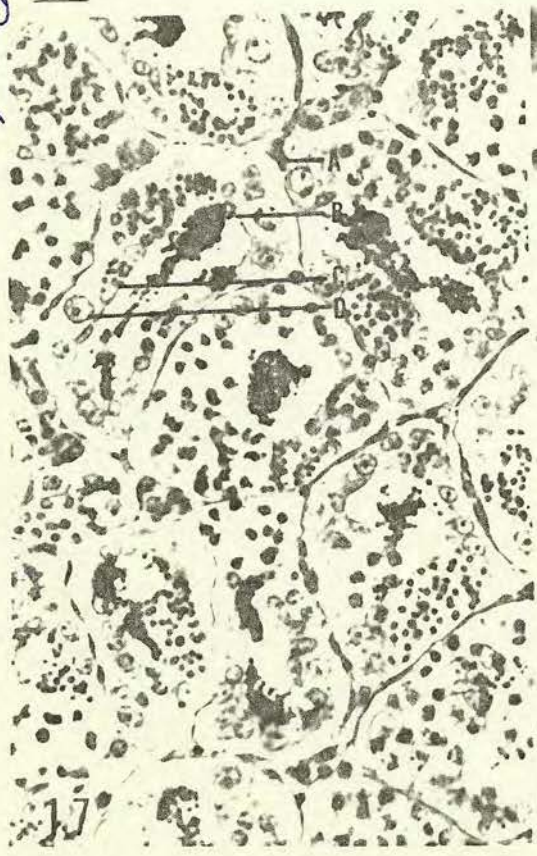

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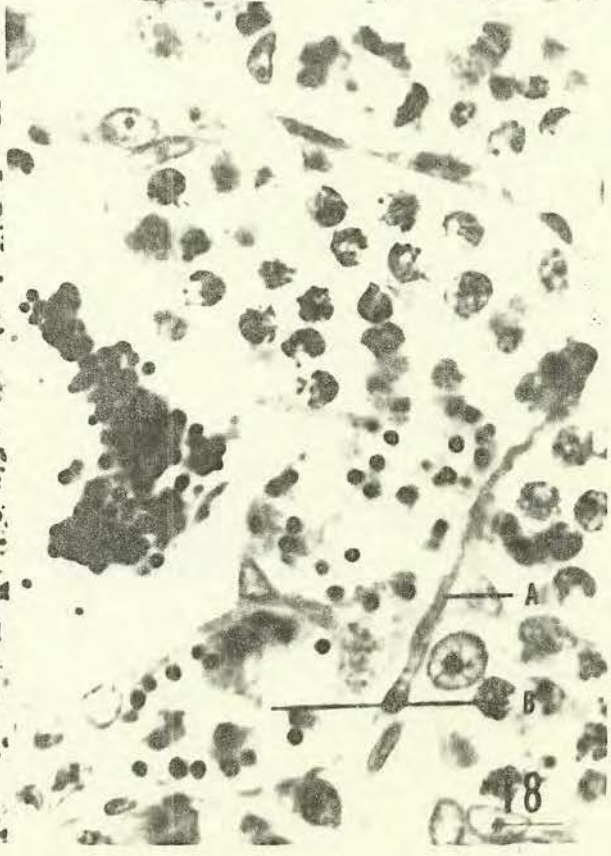

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5 weeks. Additionally, no differences in testicular germinal and stromal tissues between normal and "atypical" males were observed (Figures 16 and 17).

Onitake (1972) reported an acceleration of gonadal development in genetic female medaka with estrone treatment, while Takahashi and Takano (1971, cited by Onitake, 1972) stated ethinylestradiol suppressed proliferation and oogenetic process of germ cells in goldfish. Several other investigators, testing estrogens with teleosts, including Taylor (1948), Tavolga (1949), Ashby (1965) and Eckstein and Spira (1965), have likewise reported powerful inhibition or destruction in germinal tissue. It has been suggested that strong doses of heterologous sex steroids cause inhibition and degeneration in gonads due to their action on the pituitary by suppressing production or release of gonadotropins (Aida, 1936; Atz, 1964; Basu, 1968). These abnormal signs were not evident in this study, suggesting that the dosage levels and/or estrogens tested were estrogenically subthreshold to elicit an atypical response or were administered outside the sensitive stage when modification can be achieved.

A noted proliferation of tissue in intact testes was observed in some treatment groups and an ovotestis condition was speculated. Several investigators have reported cephalocaudal gonadal differentiation in young teleosts (Johnston, 1951; Nakamura and Takahashi, 1973). In addition, Polder (1971) noted that in cichlid intersexes, ovarian tissue is located cranially and testicular tissue caudally. On histological examination, no

ovotestis condition prevailed. Nakamura and Takahashi (1973) illustrated a stromal elongation in testes of estrogen-treated T. mossambica which may be related to the observed phenomenon.

Similar oocyte-like elements observed in testes of S. aureus specimens have been reported in other adult gonochoristic teleosts, including trout, largemouth bass, goldfish, mullet and medaka (Stenger, 1959). In mullet, the oocyte-like cells resemble true oocytes, develop in the same cyst with sperm and are distributed throughout and concentrated in the peripheral stroma (Stenger, 1959). D'Ancona (1945) mentioned that these atypical elements in eels and some other higher vertebrates are formed directly from primary germ cells without the usual sequence of oogenesis. Peters (1975) reported the occurrence of oocytes in testes of several cichlid species of the Mbuna group. All males examined were "hermaphroditic", insofar as the presence of oocytes, which varied numerically between species and individuals. No oocytes undergoing vitellogenesis were observed which prompted Peters to assume that hermaphroditic males do not transform into sexually functioning females. Oocytes possessed a similar large individual nucleolus and were found isolated along the walls of lobule tubuli. Oocytes in this study were also of comparable size to those reported by Peters (1975). He further mentioned occasional presence of small oocytes in testes of tilapia genera but did not mention the species. Polder (1971) also reported intersexes in wild populations of a cichlid fish. Hyder (1969) reported the absence of genuine cases of ovotestes in the several tilapia genera he had

examined histologically, but mentioned their observance by other investigators in personal communication. Pseudo-hermaphroditic T. aurea were disclosed by Avtalion and Mires (1976) when 18 mo-old morphologically apparent males were discovered by electrophoresis to lack the male sex marker. Dissection of these specimens revealed a large mature ovary, whereas the female genital orifice was absent. They suggested that possible sex inversion had occurred.

Common to other cichlid reports, no cases of "hermaphroditic" females were observed in this study. This is believed the first documented report of oocyte-like structures in testes of S. aureus. This phenomenon is thought not attributed to estrogen treatment as pseudo-oocytes were present in equal abundance in control groups. Their origin and function are presently unknown or at most speculative.

The finding of oocyte-like structures in testes of untreated males, the report of S. aureus apparent males (UGP) with well-developed ovaries and significantly skewed male progenies in normal intraspecific matings, all suggest a possible plasticity or unbalance in the determination of the phenotypic sexual traits in this species. The potentiality exists that the male determining mechanisms or factors predominate, causing their prevailing expression and more pronounced resistance to change. Some of the recent reports also raise questions concerning S. aureus being a true gonochorist.

Early ontological observations

Eggs undergoing early cell division, from the buccal cavity of a female and artificially incubated at 25°C, began hatching 54 hr after their collection. Almost all larvae had hatched after 57 hr. Hatched fry with prominent yolk sacs were 5.0 mm in total length. They began actively seeking food 3.5 days post-hatching when 6.5 mm and still with remnants of yolk sac. Moving food particles were more readily consumed than motionless material. The embryonic yolk sac appeared completely absorbed in 7 mm post-larvae, between 6-7 days after hatching. On absorption of yolk sac and development of swim bladder, fry became very active and consumed both stationary and moving food particles. They accepted finely ground artificial diet at approximately 6.5-6.8 mm, or about 4.5 days post-hatching. A regression of weight on length for fry 5-27 mm gave the equation $\log W = 2.61 \log L - 4.27$ (Figure 19). Growth curves in both length and weight from age 4 to 20 day-old fry after hatching are presented in Figure 20. The weight gain per day accelerated after absorption of yolk sac and transition to normal feeding activity. Results suggest that S. aureus fry of approximately 6.5 mm would consume medicated diets if treatment at this size is warranted. These observations are from laboratory reared fry and application of data to naturally-reared fry could be misleading unless experimentally proven otherwise, however, it is doubtful that growth would depart much in the earlier stages of development.

Figure 19. Length-Weight Relationship of *S. aureus* Fry 5-30 mm in Total Length

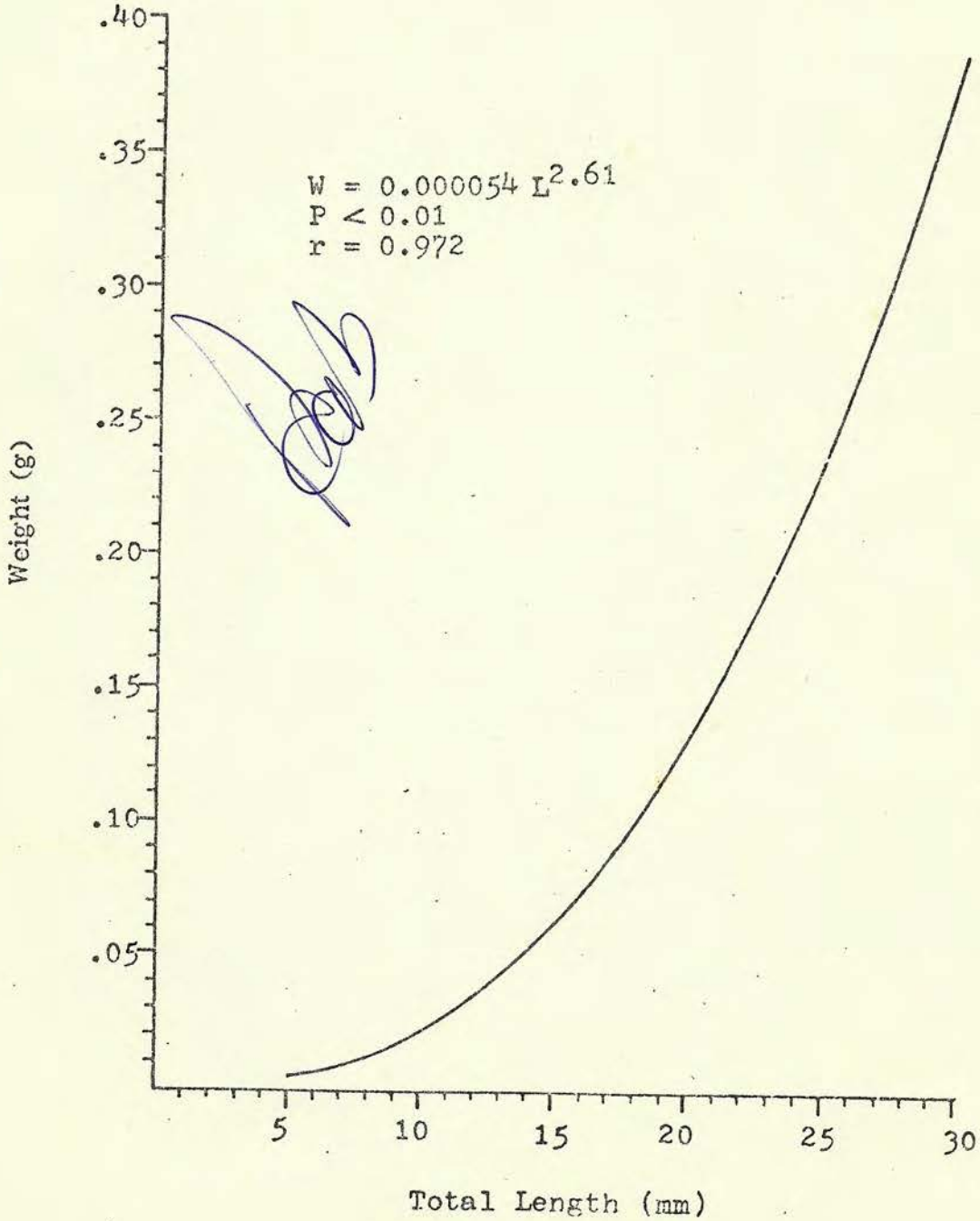
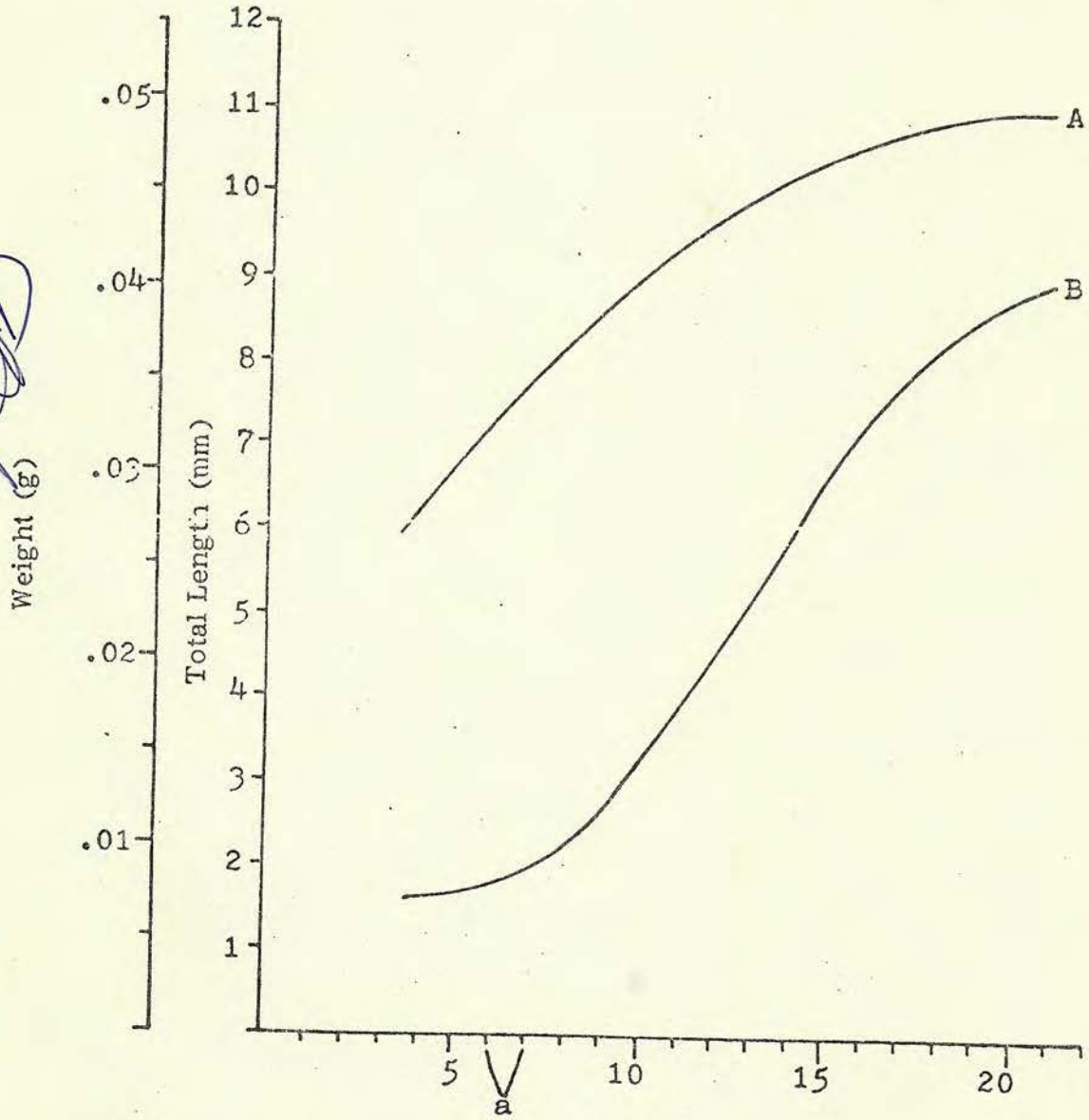


Figure 20. Growth Curves in Length and Weight for Laboratory-reared S. aureus from Hatching to 3 Weeks Post-hatching

A- Length, $Y = 3.69 + 0.69X - 0.01X^2 - 0.00007X^3$
B- Weight, $Y = 0.02 - 0.004X + 0.001X^2 - 0.00001X^3$



Age (days post-hatching)
Temperature = 25°C

[a]completion of yolk sac absorption

Mires (1974) reported T. aurea usually begin to hatch at end of the fourth or fifth day after oral incubation and by end of the eighth day accept artificial feed. Fishelson (1966) observed T. aurea fry feeding while still possessing yolk reserves and their expulsion from brooding females after 14 days at 25-27°C. It appears the first 4-5 wk of life are most critical for T. mossambica fry and that stocking outdoors delays their acceptance of prepared feed for 2 wk (Uchida and King, 1962). Cannibalism has also been observed in T. mossambica fry as small as 20 mm when stocked in outdoor rearing tanks (Uchida and King, 1962). Rothbard and Pruginin (1975) stated T. aurea and T. nilotica eggs hatch 50 h after fertilization in artificial incubators and the yolk sac is completely absorbed in an additional 8-10 days. Absorption of yolk sac 5-6 days after hatching in T. aurea at 27-30°C was reported by Mires (1973). In aquaria, McBay (1960) mentioned that T. aurea fry were 9 mm in 8 days after egg fertilization at which time remnants of yolk sac persisted and were completely absorbed in 13 and 14 day-old fry. Uchida and King (1962) reported T. mossambica fry were 5 mm at hatching, 5.8 mm on second day and 8 mm at end of eighth day after hatching. T. macrocephala fry are 5.3 mm on hatching, and like T. aurea, are unable to swim effectively due to the large yolk sac and undeveloped swim bladder (Shaw and Aronson, 1954). The egg and embryo developmental stages in S. aureus are probably similar to those illustrated by Shaw and Aronson (1954) in T. macrocephala. The

ontological observations conform with those reported by other investigators with some variation due to temperature and rearing differences.

Economic analysis of estrogen use in tilapia culture

The operations involved in potential attainment of monosex male S. aureus fingerlings with estrogens and their variable costs are presented in Table 13. Large initial fry production (Operation I) is unnecessary, requiring only several hundred fry for first estrogen treatment with the objective of producing brood fish which would be part of parental stock producing all-male offspring. Spawnings could be conducted in aquaria or small outdoor tanks. Selected fry would be transferred to indoor facilities for hormone administration (Operation II). The treatment phase (Operation III) would presumably last 4 wk, using DES as the estrogen sex reversal agent, primarily because of its low cost. Other estrogens could be substituted but their cost would be more (United States Biochemical Corporation, 1975). Depending on rearing environment, Terramycin treatment could be eliminated. After DES treatment, fry would be stocked outdoors (Operation IV) in earthen ponds at 5,000/ha to attain mature size after 6 mo (Bowman, 1974). Operation V involves identification of genetic males from all-phenotypic female group by progeny testing. This procedure would last 2 mo with assumptions of undelayed matings and sexing of subsamples of young offsprings by a gonadal squash technique (Guerrero and Shelton, 1974). Operation VI involves repeat spawnings of

TABLE 13

VARIABLE COSTS INCURRED IN HYPOTHETICAL PRODUCTION OF SEX REVERSED BROOD FISH WITH POTENTIAL OF PRODUCING MONOSEX PROGENIES

Operation / Item	Unit	No. Units	Investment Cost/Unit	Total Cost($)	Total($)
I. Fry production					
Brood fish (2♂ : 20♀)	kg	2.3	1.10	2.50	
Labor (feeding/maintenance)	hr	30	2.50	75.00	
Feed (Purina Trout Chow)	--	--	--	ns[1]	
Subtotal					77.50
II. Fry collection and stocking for estrogen administration					
Labor	hr	10	2.50	25.00	
Subtotal					25.00
III. Treatment phase (4 wk)					
Medicated feed (100 g) diethylstilbestrol @ 200ug/ g diet (DES)	g	0.02	0.21	ns	
95% Etoh	liter	0.1	1.15	.12	
Terramycin	g	5	5.00	25.00	

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TABLE 13--Cont.

VARIABLE COSTS INCURRED IN HYPOTHETICAL PRODUCTION OF SEX REVERSED BROOD FISH WITH POTENTIAL OF PRODUCING MONOSEX PROGENIES

Operation Item	Unit	No. Units	Investment Cost/Unit	Total Cost($)	Total($)
Feed	--	--	--	ns	
Labor (feeding/maintenance)	hr	42	2.50	105.00	
Subtotal					130.12
IV. Post-treatment growth to brood fish size, 18-20 cm (6 mo)					
Pond fertilizer	application	5	1.10	5.50	
Feed (Purina Trout Chow)	kg	190	.35	66.50	
Labor (feeding/maint.)	hr	135	2.50	337.50	
Subtotal					409.50
V. Progency testing and ID Estrogen-induced females (2 mo)					
Labor (feeding, maint., sexing offsprings)	hr	55	2.50	137.50	

TABLE 13-Cont.

VARIABLE COSTS INCURRED IN HYPOTHETICAL PRODUCTION OF SEX REVERSED BROOD FISH WITH POTENTIAL OF PRODUCING MONOSEX PROGENIES

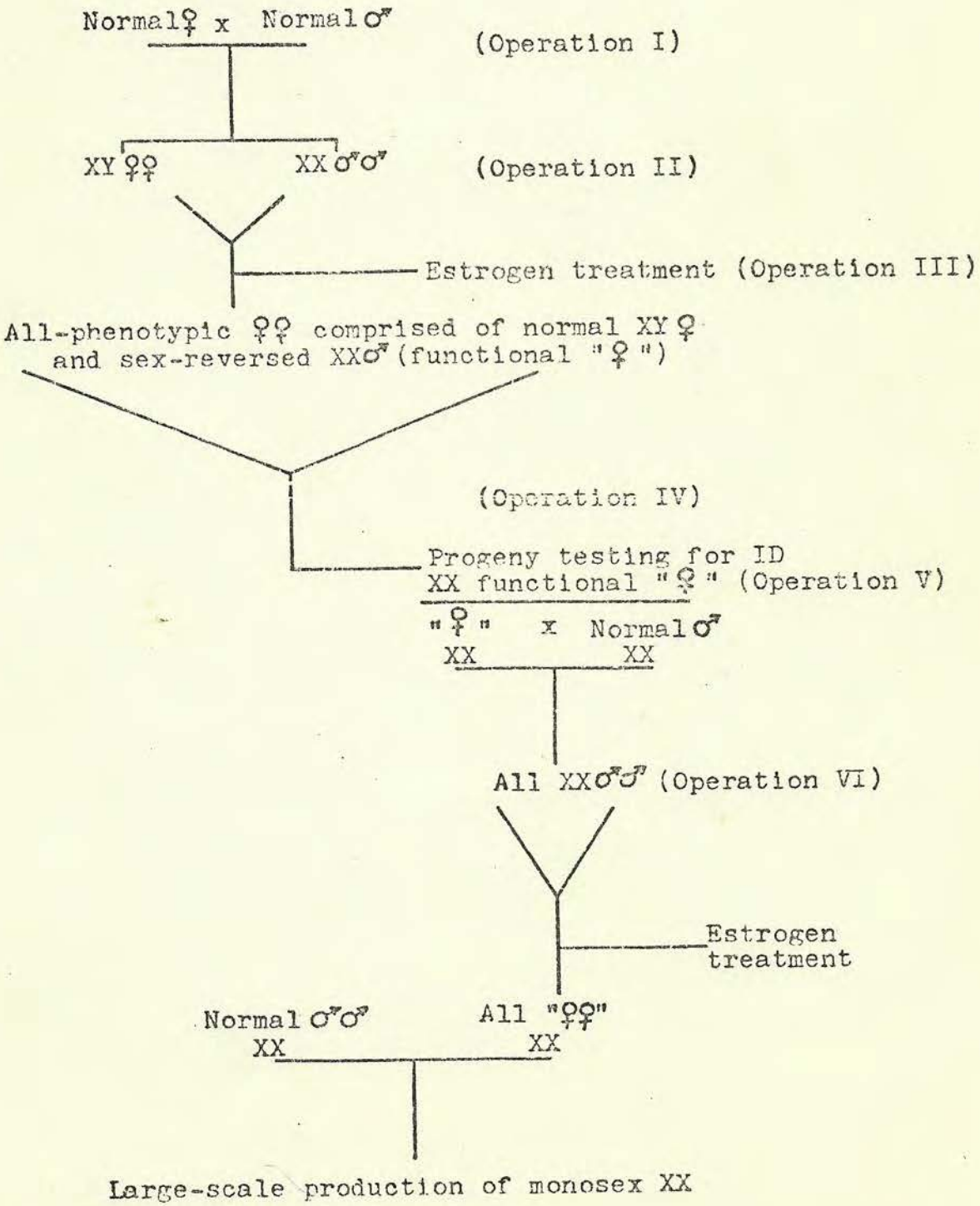
Operation Item	Unit	No. Units	Investment Cost/Unit	Total Cost($)	Total($)
Feed	kg	10	.35	3.50	
Subtotal					141.00
VI. Estrogen-induced female recruitment (500-750 fish)					
Repeat operations II-IV at same costs	--	--	--	--	
Subtotal					564.62
Total					1347.74
10% for miscellaneous items					134.77
12% interest on operating capital					177.90
Total variable costs excluding utilities and taxes to produce 500-750 sex reversed brooders capable producing some 175,000-260,000 monosex tilapia per spawn of all females					1660.41

[1]ns means not significant

identified or confirmed sex reversed brooders and sex reversal of their all-male offsprings. Procedures and their corresponding costs in Operations II-IV would be repeated on this monosex group to include their sex reversal and growth to sexual maturity. This would provide large numbers of brooders for large-scale monosex production. A flow diagram presenting the various matings and operations entailed in commercial monosex male production using estrogens is presented in Figure 21. Total variable costs to produce 500-750 sex reversed brooders would be approximately $1,660.41 or $3.32-2.21 per fish. Cost per brooder could be decreased by treating larger numbers. Assuming a mean fertility of 350 fry per brooder, total monosex fry production would be 170,000 - 260,000 per spawn at a cost of $.006-.01 each. Cost per fry on subsequent spawns of DES-induced females would be greatly reduced due to cost elimination of all operations, excluding labor in feeding, maintenance, fry collecting and fingerling rearing, plus feed costs.

In northern latitudes, T. aurea spawns three to four times annually (Yashouv, 1958; Dadzie 1970), but in perennially warmer climates or environments, spawning frequency may increase to five or six times (McBay, 1961). Size of tilapia brood also increases with size of female (Lowe, 1955b; Welcomme, 1967) and subsequent spawnings (McBay, 1961). Once sex-reversed brooders have been established, their previously undesirable fertility could be utilized to produce large numbers of valuable all-male seedlings.

Figure 21. Sexual Genotypic Matings between Estrogen-induced Females and Untreated Males of *S. aureus* and Intervening Operations for Potential Large-scale Monosex Male Productions



Cost per monosex hybrid fry of 0.009¢ from an interspecific tilapia mating in Brasil (Greenfield et al., 1974) is comparable to that for monosex production with estrogens. Pruginin and Shell (1962) stated that 500-800 T. aurea could be hand-sexed per man-day of which about one half are males. This method would cost $0.05-0.08 per male fingerling in labor costs alone. Guerrero (1974) reported a cost less than $0.50 for feed and androgen to treat 1,000 T. aurea fry with a sex steroid to produce monosex groups (excluding labor and operational expenses). As concerns commercial fry production and rearing, Hida et al. (1962) analyzed the operational costs in an intensive system and stated that one half-time operator could manage the production of 1 million fry annually.

To interpret this economic feasibility analysis in its realistic prospective, several disputable assumptions need to be stated. Most importantly is that sex reversal can be induced in genetic males of S. aureus. This point is discussed in the following section. The sex determining mechanism is stable such that intraspecific presumptive homogametic matings produce all-male offsprings consistently. Fecundity and fertility are not negatively affected by estrogen treatment. Finally, estrogen-induced females are fertile and breed naturally with untreated males. If these assumptions are proven valid, then from an economic viewpoint, estrogen use in tilapia culture would be feasible.

Potential application of estrogens in tilapia culture

Tilapia species have been distributed throughout the world in tropical and semi-tropical regions where their establishment is permanent. To further contribute extensively to fish cultural practices and food production, their monosex culture is fundamental. Naturally occurring or synthetic estrogens administered at proper concentrations could presumably induce functional feminization in homogametic genotypic males, which when mated to untreated genetic males, could theoretically produce monosex male progenies.

Several disadvantages of alternative monosex production approaches have been mentioned in the introduction. The present obvious objections to estrogen practice are its questionable feasibility and medical consideration if directly treated fry are consumed. Two facts that support eventual success in reversing sexual differentiation of male S. aureus include: (1) three gonochorist fish species have been functionally sex reversed in both sex directions with heterotypic sex hormones (Yamamoto 1953, 1958; Yamamoto and Kajishima, 1968), including a closely related cichlid, T. mossambica (Clemens and Inslee, 1968; Nakamura and Takahashi, 1973); and (2) female T. aurea have recently been completely masculinized with androgens (Guerrero, 1975; Sanico, 1975). Questions concerning the human consumption of sex-hormone-treated fish would be resolved as cultured monosex food fish do not receive steroid medication, only their parental stocks.

The action of segregating and reclaiming sex reversed brood fish from untreated stocks could be problematic, necessitating conscientious personnel to prevent their intermixing. Special indoor rearing facilities would be requisite, in addition to trained staff to conduct estrogen treatment when required. Identification of genetic males from females in all-phenotypic female groups is another inconvenience following initial medication. Progeny testing of paired matings of treated and untreated fish is currently employed, but it is time consuming, requires facilities to conduct controlled spawnings and delays recognition of desired brooders. Cytological chromosome studies and electrophoretic techniques offer some promise in expediting this distinction. Fortunately, once a few sex reversed brooders have been identified, their all-genetic male progenies could then be sex reversed. This would eliminate the need for additional progeny testing and provide large numbers of potential brooders for large-scale monosex production.

This method has several advantages which advocate its potential workable practice. The operation is primarily labor-intensive and in countries with low labor costs, capital outlay would be minimal. Expenditure for estrogenic material is also inexpensive. Following initial delays in procuring sex reversed individuals and first generation treatment of their monosex offsprings, additional medication would be infrequent, i.e., only to replace sex reversed brooders every 1-2 years. Possible

detrimental effects from an inbreeding population could be alleviated by introducing new stocks of untreated males.

Use of estrogens could be applicable to homogametic tilapia males which include T. hornorum, T. macrochir, Tilapia variabilis and T. aurea. This being factual if the coexistence of heterogametic males and females exists in tilapia species, as discussed and evaluated by Chen (1969) and Jalaberi et al. (1971). In countries or areas reluctant to introduce another tilapia species for monosex hybrid productions for fear of displacing endemic fishes, estrogen application could substitute as a valuable tool in management of existing species. Marginal cold tolerance areas could be more extensively utilized by monosex S. aureus, most cold resistant of presently cultured tilapias, without possibility of removing or diluting this trait through hybridization.

Of the diverse tilapia culture techniques proposed and practiced, each has its unique advantages under specific conditions, dependent on objectives of culture operation. Use of estrogens provides yet another potentially viable alternative to their successful management.

V. SUMMARY AND CONCLUSIONS

Brood Spawning and Fry Procurement

1. Stocking 225 1 year-old S. aureus brooders at 2♀ :1♂ in a 0.04 ha earthen pond (5625/ha), produced in excess of the needed 3500, 8-11 mm fry for experimental use.

2. Brood fish were stocked 1 May, spawning took place during the second week after stocking and females were observed carrying eggs or yolk-sac fry on 17 May.

3. Experimental fry were gathered from 23-25 May, which proved to be about 7 days late, as many collected fry were larger (greater than 11 mm) than the acceptable size.

4. Fry 8-11 mm were estimated to be approximately 8-21 days-old post-hatching.

5. Out-of-season spawning in controlled overwintering facilities suggests possible year-round tilapia fry production in regulated environments.

Treatment Phase

1. Groups of 8-11 mm total length S. aureus fry were treated perorally with estriol, estrone and 17B-estradiol each at concentrations

of 30 ug/, 60 ug/ and 120 ug/ g artificial diet for both 3 and 5 wk periods.

2. Crowding of fry at 1620/m^2 in continuous water flow steel troughs produced active feeding, enabled minimum fry handling, but retarded growth somewhat compared to lesser stocking densities (less than 463/m^2).

3. Fry reached mean total lengths of 17.7 mm and 25.3 mm after 3 and 5 treatment weeks, respectively. Nonsignifcant trends in growth differences between experimental and control groups indicated no adverse treatment effects during medication administered periods.

4. Mean over-all fry recovery in experimental groups was 94.2% and did not differ significantly from the control (93.4%), denoting no lethal effects from steroid treatments. The majority (88.1%) of the unrecovered fry were not mortalities, but fry that escaped their enclosures and exited via the drainpipe.

Post-treatment Growth Phase

1. Experimental groups were stocked in 0.002 outdoor concrete tanks at 43,000-101,500/ha, with a mean of 64,250/ha, for 106 and 127 mean growth days for 3-and 5-wk treatment groups, respectively.

2. The mean length and weight for 3-and 5-wk treatment groups were 145 mm, 51.1 g and 128 mm, 34.7 g, respectively. Growth was influenced by the number of favorable temperature culture days.

3. The mean recoveries for 3 and 5 wk estrogen-treated groups were 81.5% and 87.2%, respectively. Recoveries in most treatment groups were comparable to those of controls. Treatments with lower recovery rates either experienced critical oxygen depletions and/or had many unaccounted fish. Significant mortalities in some treatments were caused by bacterial epizootics.

4. No delayed lethal or adverse effects from estrogen administration were evidenced during growth phase.

5. A high proportion (23.8%) of fish at harvest had physical deformities or atypical features and experienced inferior growth. Their occurrence was equally distributed among experimental and control groups. Poor water quality during rearing could account for some anomalies, but genetic inheritance from parental stocks is also suspected.

6. A correlated trend existed between recovered mortalities and recorded water temperatures which revealed a seasonal periodicity of mortalities.

7. The gonadal sexing of 97 recovered mortalities indicated no significant sexual differential in mortalities.

8. A bimodal size distribution after hormonal treatment suggested a possible early sexual growth difference. Analysis of sex ratios from groups of two size categories indicated no significant difference in sex distribution between the two size classes.

9. No reproduction was observed during the growth phase of the treatment and control groups.

Sex Ratios and Sex Reversal

1. The three estrogens administered at varying concentrations and time regimes did not significantly alter sex ratios of treated S. aureus fry.

2. There were significant differences in the distribution and occurrence of females between the 3 and 5-wk treatment periods evaluated.

3. No significant trends were observed in female proportions within different dosage levels, separate and combined, for each estrogen individually in both 3-and 5-wk treatment regimes. Similar nonsignificant findings in female ratios resulted between combined female proportions in similar dosage levels for all estrogens and between treatments and controls for both treatment periods.

4. Of all recovered estrogen-treated fish with testes, 34.2% possessed female-like urogenital papillae. At least 45% of the males in several treatments possessed this anomaly.

5. A trend revealed a higher proportion of "atypical" males with increasing estrogen dosage levels and potencies.

6. A significantly greater proportion of "atypical" males occurred in groups of the longer (5 wk) treatment period, indicating a superior response to extended exposure to medication.

7. Failure to induce complete sex reversal in S. aureus fry is believed due to an insufficient estrogen potency, and/or, more probably, an inadequate treatment period.

Histological Examination of Gonads

1. No obvious changes were observed in sectioned and stained gonads examined from estrogen-treated fish of both sexes.

2. Normal spermatogenetic and oogenetic processes were neither suppressed nor accelerated by steroid treatment.

3. Pseudo-oocyte elements similar to female primary oocytes occurred in testes of fish from both experimental and control groups.

4. No ovotestis condition was observed in gonads of fish sampled.

5. Noneffective alteration of testis by estrogens tested, suggests insufficient concentrations required to modify or completely reverse gonad phenotype or possible treatment outside the critical period of gonad sexual determination and differentiation.

6. The occurrence of pseudo-oocytes in testes of S. aureus males and recent reports of aberrant sex ratios and male-appearing fish with developed ovaries, might indicate a sexual imbalance in potentiality of phenotypic sex expression and questionability of the gonochoristic status of this species.

Early Ontological Observations

1. S. aureus pro-larvae were 5.0 mm in total length after hatching.

2. Pro-larvae were observed to feed on exogenous artificial feed 3.5 days after hatching or when 6.5 mm.

3. The embryonic yolk sac was completely absorbed in 6.5-6.8 mm fry, 6-7 days after hatching.

4. A regression of weight on length for S. aureus fry 5.0-27 mm gave the equation log W = 2.52 log L - 4.25.

5. Growth curves of laboratory-reared S. aureus fry from hatching to 3 wk post-hatching were from third degree polynomial equations of $Y = 3.69 + 0.69x - 0.01x^2 - 0.00007x^3$ and $Y = 0.02 - 0.004x + 0.001x^2 - 0.00001x^3$ for length and weight, respectively, vs. age.

Economic Analysis of Estrogen Use in Tilapia Culture

1. The operations involved in potential large-scale production of S. aureus monosex males and their corresponding variable costs were discussed and presented.

2. The estimated total cost to produce 500-750 sex-reversed S. aureus brooders was $1,660.41 or $3.32-2.21 per fish and $.006-.01 per monosex male fingerling produced from selected matings.

3. A diagram was presented which depicted the various chosen matings with their presumed sexual genotypes and the intervening operations used for potential commercial production of monosex males.

4. Use of estrogens to produce monosex S. aureus males appears economically feasible and is comparable to costs involved in employing

hand-sexing and interspecific hybridization practices.

5. Leading assumptions, yet unrealized or unproven, in estrogen implementation include; complete sex reversal of homogametic tilapia males can be achieved, the sex determining mechanism is stable enough to allow consistent monosex offspring production, fertility and fecundity are not detrimentally affected by estrogen treatment, and selected matings occur naturally and readily.

Potential Application of Estrogens in Tilapia Culture

1. Several established results and facts support the eventual success in complete, functional sex inversion of male S. aureus with estrogen administration.

2. Questions and skepticism concerning the consumption of sex hormone treated fish are dismissed as monosex male offspring do not directly receive estrogen medication, only their estrogen-induced female parents.

3. Once sex-reversed brooders are confirmed, further operations are reduced, allowing direct production of monosex males.

4. Estrogen treatment could be potentially applicable to all homogametic males of the genus Tilapia, which presently includes several economically important pond fish.

5. Monosex S. aureus males could be obtained without serious alteration or dilution of their valuable cold-resistant trait which could occur through interspecific or intergeneric matings.

6. Areas with only one tilapia species in which the male is homogametic sex, can potentially obtain monosex males without need to hand sex or introduce new species to enable production of monosex hybrids.

7. All growth and culture advantages of rearing all-male stocks would be realized and would thus enhance support for estrogen use.

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APPENDIX

TABLE 14

COMPARISON OF RECOVERY DATA BETWEEN TREATMENTS ON HARVEST FROM GROWTH TANKS[1]

Treatment	Total No. Stocked	Total No. Mortalities Recorded	Total No. Unaccounted	Total No. Recovered	% Total Recovery
Control	327	18	15	294	89.9
Estriol 30	337	20	28	289	85.8
Estriol 60	375	7	21	347	92.5
Estriol 120	327	32	75	220	67.3
Estrone 30	340	35	65	240	70.6
Estrone 60	329	6	27	296	90.0
Estrone 120	295	3	67	225	76.3
Estradiol 30	341	8	30	303	88.9
Estradiol 60	325	14	26	285	87.7
Estradiol 120	345	14	14	317	91.9

[1]Combined data from 3-and 5-week treatment groups

TABLE 15

SUMMARY OF CUMULATIVE PERCENT RECOVERY AT END OF TREATMENT AND GROWTH PHASES[1]

Treatment	Initial %	% Recovery End Treatment	% Recovery End Growth
Control	100 (350)[2]	93.4 (327)	84.0 (294)
Estriol 30	100 (350)	96.3 (337)	82.6 (289)
Estriol 60	100 (400)	93.7 (375)	86.7 (347)
Estriol 120	100 (350)	93.4 (327)	62.9 (220)
Estrone 30	100 (350)	97.1 (340)	68.6 (240)
Estrone 60	100 (350)	94.0 (329)	84.6 (296)
Estrone 120	100 (350)	84.3 (295)	64.3 (225)
Estradiol 30	100 (350)	97.4 (343)	86.6 (303)
Estradiol 60	100 (350)	92.9 (325)	81.4 (285)
Estradiol 120	100 (350)	98.6 (345)	90.5 (317)

[1]Combined data from 3-and 5-wk treatment groups

[2]Number of fish

TABLE 16

COMPARISON OF MEAN WEIGHTS AND LENGTHS OF MALES AND FEMALES IN 3-WEEK TREATMENT GROUPS STOCKED IN 0.002 ha CONCRETE TANKS

Treatment	Males			Females		
	Wt. (g)	Length (mm)	s.d.[1] (mm)	Wt. (g)	Length (mm)	s.d. (mm)
Control	49.5	142	13.4	41.3	136	12.0
Estriol 30	74.9	160	9.4	51.9	144	11.0
Estriol 60	66.2	160	10.7	59.8	147	10.2
Estriol 120	55.7	149	10.2	42.9	138	12.0
Estrone 30	67.6	155	6.8	49.3	141	12.6
Estrone 60	48.6	149	19.1	39.0	137	17.6
Estrone 120	27.7	134	15.2	28.1	124	13.0
Estradiol 30	67.2	154	11.0	50.0	144	13.5
Estradiol 60	72.4	157	15.8	47.5	145	10.6
Estradiol 120	54.5	152	17.4	46.0	143	14.4

[1] s.d. refers to standard deviation

TABLE 17

COMPARISON OF MEAN WEIGHTS AND LENGTHS OF MALES AND FEMALES IN 5-WEEK TREATMENT GROUPS STOCKED IN 0.002 ha CONCRETE TANKS

Treatment	Males			Females		
	Wt. (g)	Length (mm)	s.d.[1] (mm)	Wt. (g)	Length (mm)	s.d. (mm)
Control	55.9	149	13.2	45.1	139	12.9
Estriol 30	35.9	132	13.5	25.2	119	12.8
Estriol 60	30.2	122	16.0	22.6	112	13.8
Estriol 120	37.9	132	12.7	37.9	125	9.1
Estrone 30	33.2	128	11.0	24.4	113	13.4
Estrone 60	40.9	137	11.1	29.4	125	12.1
Estrone 120	46.0	137	14.1	34.0	124	15.6
Estradiol 30	36.4	129	14.1	26.6	118	13.6
Estradiol 60	41.2	136	12.5	32.3	126	11.2
Estradiol 120	39.0	132	13.8	26.1	119	13.5

[1] s.d. refers to standard deviation